FUNCTIONAL ANALYSES OF THE NOVEL STRESS-INDUCIBLE XVPSAP PROMOTER ISOLATED FROM *XEROPHYTA VISCOSA*

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Functional analyses of the stress-inducible XvPsap promoter isolated from *Xerophyta viscosa*

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This treatise is dedicated to

my dear wife Roseline,

daughter Zawadi and son Ccoga

The world ranks you according to your own rating. If you view yourself as small and insignificant like a worm, others will go fishing with you.

Declaration

I declare that the work presented in this thesis is my original work. I also affirm that this work has not been presented in this, or any other university for examination, or for any other purposes. I further declare that the XvPsap promoter sequences have been patented and are included in this thesis with permission from the other inventors (Patent File Ref. PA143860/PCT).

OKOTH RICHARD ODUOR
FEBRUARY 2009

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Abbreviations

The following abbreviations have been used in this thesis. They are defined in Chapters where they first appear. Unless otherwise defined in the local area of the text, the definitions for the abbreviations apply throughout the whole text.

μl	Microlitre(s)
μg	Microgram(s)
μM	Micromolar(s)
°C	Degrees Celsius
$'$	Prime
α	Alpha
β	Beta
2,4-D	2,4- Dichlorophenoxy acetic acid
ANOVA	Analysis of variance
ABRE	ABA-responsive element
AS	Acetosyringone
BAP	Benzyl Amino Purine
BLAST	Basic logarithm alignment search tool
BMS	Black Mexican Sweetcorn
bp	Base pair(s)
BSA	Bovine serum albumin
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary deoxyribonucleic acid

CDP-*Star*	Disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2-(5-chloro) -tricyclo[3.3.1.1^{3,7}]decan}- 4-yl)-1-phenyl phosphate
CTAB	Cetyl trimethylammonium bromide
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxy-nucleotide triphosphate
DPE	Downstream promoter element(s)
dTTP	Deoxythiamidine triphosphate
dUTP	Deoxyuridine triphosphate
g	Centrifugal force
g	Gram
GE	Genetic engineering
GFP	Green fluorescent protein
GOI	Gene of interest(s)
h	Hour(s)
HKG	House keeping gene(s)
HSE	Heat stress responsive elements
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilobase(s)
LB	Left border
LS	Lansmaier and Skoog
MBS	MYB binding sites
MES	2-(4-morpholino)-ethane sulfonic acid
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar

mRNA	Messenger RNA
MS	Murashige and Skoog
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National center for biotechnology Information
NEB	New England Biolabs
nos	Nopaline synthase
OD	Optical density
PCR	Polymerase chain reaction
qRTPCR	Quantitative real time polymerase chain reaction
RB	Right border
RLU	Relative light unit
RNA	Ribonucleic acid
RWC	Relative water content
s	Second(s)
SSC	Saline sodium citrate
T-DNA	Transferred DNA of tumour-inducing (Ti) plasmid
TF	Transcriptional factor
TFCC	Transcription-factor-centric clustering
Triton X-100	Poly(ethylenglycolether)n-octylphenol
tPA	Tissue type plasminogen activator
TSS	Transcription start site(s)
U	Units
UV	Ultra violet
W_d	Dry weight
W_{ft}	Weight at full turgor
W_{ini}	Initial weight
w/v	Weight per volume
WT	Wild type
X-gal	5-bromo-4-chloro-3-indoyl- -D-galactopyranoside

Abstract

A non-coding sequence, XvPsap1 (2083 bp), was isolated upstream of the *XvSap1* gene of *Xerophyta viscosa*. The sequence was analysed for novelty and the existence of putative *cis*-elements. No significant identity was observed to any known plant promoter. Various putative *cis*-acting elements were identified including those involved in dehydration, heat stress, abscisic acid, jasmonic acid, light and low temperature responsiveness. Two 5′ promoter deletions were conducted resulting in truncated promoter fragments designated XvPsap2 (1577 bp) and XvPsap3 (1127 bp) relative to the translation start site. The full length promoter (XvPsap1) and truncated fragments (XvPsap2 and XvPsap3) were cloned upstream of the coding regions of either firefly luciferase or the jellyfish green fluorescent protein. The gene constructs were used to transform Black Mexican Sweetcorn (BMS) cells and *Nicotiana tabacum* plants. Only the XvPsap1 construct was used to transform *Zea mays* plants. The functional properties of each promoter fragment were assessed by fluorescence quantitative analyses. The transgenic BMS cells were assessed following 72 h salt stress whereas transgenic tobacco and maize were dehydrated for 8 days. Plants or cells transformed with XvPsap1 promoter fragments displayed high luciferase activity whereas transformants of XvPsap3 demonstrated the lowest activity in both transgenic BMS cells and tobacco. Quantitative real time PCR analysis of the BMS cells indicated that the *luc* transgene was upregulated within 24 h of salt stress for both XvPsap1 (5.9-fold; $P < 0.05$) and XvPsap2 (1.9-fold; $P < 0.05$) whereas with XvPsap3 (1.9-fold; $P < 0.05$) upregulation occurred after 48 h. A similar trend was observed with dehydrated tobacco, in which optimal

expression levels were recorded three days after initiating dehydration. The increase in XvPsap1 activity was significant (7-fold; $P < 0.05$) in transgenic tobacco whereas XvPsap2 and XvPsap3 displayed minimal increases of 2.2- and 1.6-fold, respectively. In dehydrated maize transformed with XvPsap1, a significant peak in activity was observed on the third day with a 4-fold increase in luciferase expression. Together, these results suggest that the XvPsap1 promoter is stress-inducible. Furthermore, it is the most active of the three sequences assessed. It is postulated that the promoter is involved in early responses to drought as it peaks shortly after transgenic plants are subjected to lack of water. This XvPsap1 promoter could therefore find application in the generation of transgenic drought tolerant plants. Accordingly, and in a separate study, generation of transgenic maize which are drought tolerant has now been initiated using constructs containing drought tolerant genes regulated by the XvPsap1 promoter.

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Chapter 1

Introduction and Literature review

1.1 Introduction

The world's food requirements are expected to double by 2025 as a result of the projected increase in the world's population from the current 6 billion to about 7.5 billion by 2020 (Rosegrant et al. 2001). This increased global population will invariably result in increased demand for food, exacerbated by erratic rainfall, salinity and reduced arable land (Ericksen 2008). In addition, global warming has resulted in a progressive decline in crop yield due to increased water shortage (IPCC 2007). Interestingly, about 97.5% of this increase is expected to occur in today's developing world where three of every four people live in rural areas and depend directly or indirectly on agriculture for their livelihoods (Pardey & Wright 2003).

Due to the decrease in yield, the global grain markets are now facing breaking point. For instance, experts predict that if China's recent urbanisation trends continue, and the country imports just 5% more of its grain, the world's grain export would be entirely used up (Simelton et al. 2009). The knock-on effect on food supply, and on prices, to developing nations would be huge. Therefore, scientists and farmers particularly those in Africa, must now embrace new technologies in order to feed

their people (Machuka 2001, Thomson 2002).

Various strategies, including conventional breeding (Edmeades et al. 1999), have been employed in the past to develop drought tolerant plants capable of increased productivity in dry environments. However, limited success has been achieved suggesting the need to complement such activities with genetic transformation. In the Molecular and Cellular Biology laboratory at the University of Cape Town, genes conferring abiotic stress tolerance have been isolated and characterized from the resurrection plant, *Xerophyta viscosa* (Mundree & Farrant 2000, Mowla et al. 2002, Garwe et al. 2003, Govender 2006). An example of such a gene is *XvSap1*, which was observed to confer drought tolerance to transgenic *Arabidopis thaliana* and *Nicotian tabacum* (Garwe et al. 2006).

Success in manipulating these genes may find application in improving tolerance of higher plants to various abiotic stresses. However, when such genes are constitutively expressed, drought tolerance is usually accompanied with unwanted phenotypes such as dwarfism (Su et al. 1998, Hsieh et al. 2002a,b). This study sought to assess the activity of a putative stress-inducible promoter that naturally regulates the expression of *XvSap1* gene in *X. viscosa*. Furthermore, functional analysis of the promoter in various transgenic biosystems such as maize suspension cells, tobacco and maize was also determined.

1.2 Production and consumption of maize

Maize is currently a widely grown cereal crop and is third (Fig. 1.1) after wheat and rice in area harvested and total production (DeVries & Toenniessen 2001). Globally, 21 % of total grain production is consumed as food with developing countries taking the greater percentage. Industrialised countries dominate the production of maize because production is generally concentrated in zones of abundant rainfall and fertile soils (Morris 1998) and the use of many inputs and technology is extensive (Pollak & White 1995).

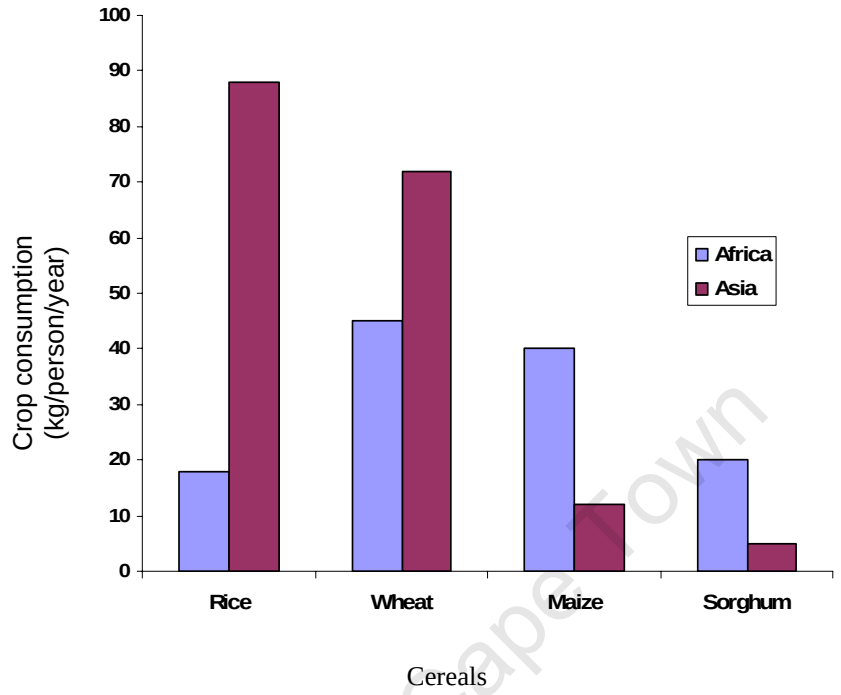

Figure 1.1: Cereal crop consumption trends in Asia and Africa, 1997 (Source: DeVries and Toenniessen 2001)

In developing countries especially those in sub-Saharan Africa, use of maize is variable. One of its main uses is for food (Table 1.1). With Africa's population expected to increase, so is the demand for imported food, mostly cereals and legumes, projected to rise from 50 to 70 million tons per year (Kelemu et al. 2003). If the current economic situation of developing countries does not improve, food-deficit nations are unlikely to have the resources to purchase such a huge volume of food on a commercial basis. Over eighty developing countries lack sufficient food to feed their populations and the money to import food supplies (DaSilva et al. 2002). Several countries are already regular recipients of food aid. Even if food aid continues, it often misses the rural poor making the approach unsustainable. To prevent future human catastrophes, African countries will have to develop and implement sustainable food production strategies for increasing agricultural productivity to enhance overall income and economic growth.

Table 1.1: Annual maize consumption per capita in selected countries in the world (Source: Morris 1998)

Country	Annual consumption of maize per capita (kg)
Malawi	137
Mexico	127
Zambia	113
Guatemala	103
Honduras	98
South Africa	94
El Salvador	93
Kenya	93
Zimbabwe	89
Lesotho	87
Venezuela	68
Nicaragua	56

1.3 Constraints to maize production

The declining trend in food productivity has been largely attributed to both biotic (Ajanga & Hillocks 2000) and abiotic constraints to crop production (Wang et al. 2003, Lea et al. 2004). Among the abiotic stresses, drought or water stress is reported to have adverse effects on crop yield resulting in average annual yield losses estimated at 17% in the tropics although values as high as 60% have been documented in individual seasons in regions such as South Africa (Sanchez et al. 2006). This yield loss is exacerbated by the fact that the most sensitive phases of crop growth to water deficit are during crop establishment and flowering (Bray

1997, Barnabas et al. 2008). Agriculture accounts for at least 70% of the world's total water usage and about 18% of the global farmland is irrigated with up to 46% of the global food supply produced on this land (Inocencio et al. 2003). Other constraints to maize productivity include environmental degradation, soil nutrient depletion, low fertilizer inputs, inadequate food processing amenities, poor roads to markets, and general lack of information to make science-based decisions that underlie farming methodologies and systems (Machuka 2001).

1.4 Maize improvement strategies

1.4.1 Conventional breeding

Breeding involves improvement through a series of recurrent selection procedures aimed at maximizing percentages of favourable alleles at each locus of importance in a given environment (Kling & Edmeades 1997, Lamkey 2002). However, conventional breeding has limitations that impede its sole adoption since it is time consuming and laborious, difficult to modify single traits and relies only on existing genetic variability (Holmberg & Bulow 1998, Zhang et al. 2000). Thus other complementary approaches such as genetic transformation are now being explored.

1.4.2 Genetic engineering

Genetic transformation can now be used as a relatively fast and precise means of achieving stress tolerance in various crops including maize. Unlike conventional breeding, advances in genetic engineering has enabled exchange of genes between sexually incompatible species thus providing a wider gene pool for manipulation. Furthermore, the ability to transfer single, specific genes by genetic engineering allows for precise transfer of minimum DNA necessary for the desired trait to be obtained. This reduces chances for linkage drag which is associated with traditional breeding in which genetically linked, undesired genes are also transferred. More-

over, genetic engineering enables the transfer of new genes directly into existing plant lines, thereby minimizing tedious time-consuming plant breeding cycles, in which many generations are required in order to recover specific lines.

1.5 Transgenic maize

First generation transgenic crops were mainly engineered against biotic constraints to crop productivity with little efforts directed on abiotic stresses. As a result, transgenic maize resistant to insect pests and herbicide were among the first crops to be reported (Pilcher et al. 1997, Lynch et al. 1999). Transgenic maize with elevated 10kd zein and methionine have also been obtained (Anthony et al. 1997). In addition, antifungal proteins, such as chitinases and beta-1, 3-glucanases, have been genetically engineered to attempt expression in the maize kernels with the aim to prevent the growth of *Aspergillus flavus* and the production of aflatoxins (Wu et al. 1994, Wan et al. 1995).

Currently, several studies are being undertaken to address drought stress. Most recently, Monsanto in collaboration with BASF have developed transgenic drought tolerant maize currently undergoing field trials in selected African countries under the water efficient maize for Africa (WEMA) programme (www.aatf.africa.org). While such efforts are laudable, the quest for developing drought tolerant maize should still continue given the polygenic nature of drought stress. The low research output in developing transgenic drought tolerant maize could partially be attributed to insufficient availability of desired genes, appropriate promoters and reliable gene delivery systems. However, since genes conferring drought tolerance have been isolated and characterised in the past especially from *X. viscosa* (Mundree & Farrant 2000, Mowla et al. 2002, Garwe et al. 2003), the challenge is how best these genes can be delivered into the preferred host and be upregulated only during stress.

1.6 Plant promoters and gene expression

The interaction between transcription factors and *cis*-acting regulatory sequences present in plant promoters is a key step involved in the regulation of plant gene expression (Yang et al. 2000). To express the coding regions of given genes in transgenic plants, a promoter is required upstream (Galun & Breiman 1996). Genetic transformation commonly involves two genes. One is the transgene that should be integrated in the plant genome and expressed in the transgenic plant and the other is a selectable gene, such as rendering a plant resistant to a herbicide. Each of the two transgenes should thus have its own promoter and the two promoters may differ. For specific purposes the transgene that serves as a reporter gene may not be preceded by any promoter. The rationale in such cases is that when the coding sequence of the reporter gene with its terminator region is integrated in the plant genome, downstream of a promoter of this genome, the reporter gene will be expressed and the expression regulated by the host-plant's promoter (Galun & Breiman 1996).

Promoters can now be selected that allow either constitutive gene expression or limit expression to specific cell types or in response to specific environmental stimuli. Under constitutive gene expression there is gene expression all the time and usually at high levels. Examples of constitutive promoters include the Cauliflower mosaic virus promoter for 35S RNA and the maize ubiquitin promoter. It has been reported that constitutive expression of proteins involved in abiotic stress resistance hampers the normal growth of transgenic plants resulting in smaller phenotypes as compared to wild type plants (Su et al. 1998). This unwanted dwarfing of the transformed plants may be due to the expression of a protein in amounts more than normal and at stages when it is not needed thus placing a metabolic burden on the plant (Liu et al. 1998).

Stress-induced promoters have been reported to have poor levels of expression when compared to constitutive promoters (Reynolds 1999). It is therefore important

that fine manipulations are carried out in such a way that the strength of stress-responsive promoters is increased without any negative impact on their induction patterns. An example of a stress-inducible promoter that has widely been reported is the *rd29A* promoter from *Arabidosis* (Kasuga et al. 2004, Shiqing et al. 2005, Pino et al. 2007). Other stress-inducible promoters such as ABRC from barley (Lee et al. 2003) and SWPA2 from sweetpotato (Kim et al. 2003) have also been reported.

1.7 Bioinformatics as a tool for promoter analysis

With recent advances in computational tools, several software tools have been developed, either for deposition and organisation of biological data and/or for the prediction of putative functions or the structure of complex molecules specifically for plants (Venter & Botha 2004). Numerous functional elements have been identified as promoter constituents for precise and regulated transcriptional initiation such as TATA box, Initiator (Inr) motif, Downstream Promoter Element (DPE, found in *Drosophila*), TFIIB-Recognition Element (BRE), and *cis*-regulatory elements (Carey & Smale 2001, Butler & Kadonaga 2002, Smale & Kadonaga 2003). Moreover, transcription start sites (TSS) in plant promoters have been reported to have a CG-compositional strand bias, or GC-skew, where C is more frequently observed in the (+) strand than G (Tatarinova et al. 2003, Fujimori et al. 2005).

The major databases for the identification of TF-binding sites and *cis*-elements in plant promoters include, PLACE (Higo et al. 1999), PlantCARE (Rombauts et al. 1999, Lescot et al. 2002), TRANSFAC (Hehl & Wingender 2001), AGRIS (Davuluri et al. 2003) and AthaMap (Steffens et al. 2004, Bulow et al. 2006). According to Venture and Botha (2004), the important promoter detection methods, based on maximum likelihood estimation, are Gibbs-sampling (Lawrence et al. 1993) and expectation maximisation (MEME) (Bailey & Elkan 1995). Gibbs-sampling and

MEME are known as probabilistic methods that perform a local optimisation on motifs in sequences to be unknown (hidden motif in noisy background sequence) in order to identify the most conserved sequence (Ohler & Niemann 2001).

Other methods used for the identification of transcriptional regulatory elements include AlignACE (together with CompareACE and ScanACE) based on a Gibbs-sampling algorithm (Hughes et al. 2000), PROMO using sequence information from the database TRANSFAC (Messeguer et al. 2002) and transcription-factor-centric clustering (TFCC) designed to link regulatory promoter elements with the binding TFs *in silico* (Zhu et al. 2002). Although most promoter studies have obtained certain success, the current general knowledge of promoters is still insufficient (Bajic et al. 2004) suggesting the need for more studies.

1.8 Plant responses to abiotic stress

The understanding of the biochemical and molecular basis by which plants respond to abiotic stresses is pivotal in the application of various crop improvement strategies including genetic engineering (Ingram & Bartels 1996). Abiotic stress induces a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). In response to such stresses, plants exhibit a wide range of defense responses at the molecular, cellular and whole plant levels (Greenway & Munns 1980, Zhu et al. 1997, Yeo 1998, Bohnert et al. 1999, Hasegawa et al. 2000, Lin et al. 2008).

Various genes are induced in response to drought at the transcriptional level with their gene products thought to function in response to drought resulting in drought tolerance (Mundree et al. 2002). Studies of expression and functions of stress-inducible genes at molecular level have revealed complex mechanisms (Fig. 1.2) involved in gene expression and signal transduction in response to drought stress (Bray 1993).

In general, plant responses are of three kinds: (i) maintenance of homeostasis; (ii) detoxification of harmful elements; and (iii) recovery of growth. All the mechanisms are probably employed for desiccation-tolerance with individual plants utilising one strategy more than the others (Oliver & Bewley 1997, Xiong & Zhu 2002). The duration and severity of the response is a function of the plant species, the stage of its growth and the type of stress.

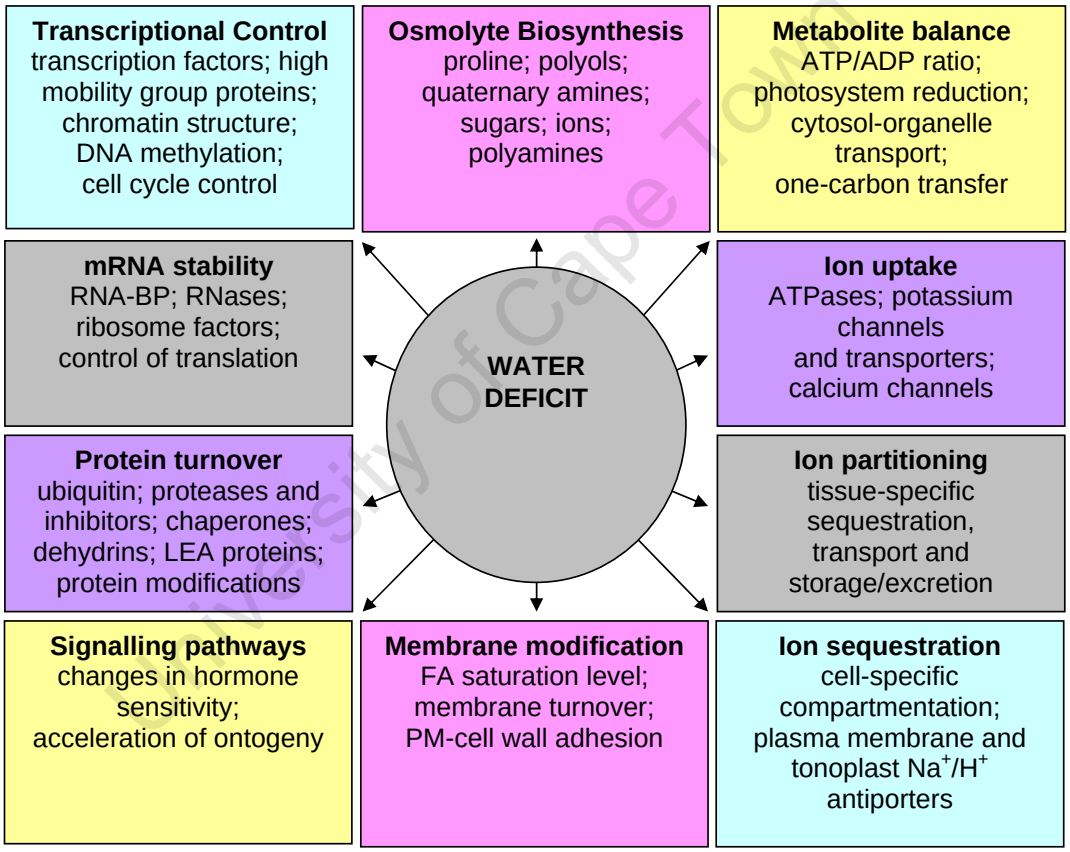

Figure 1.2: Schematic outline of plant responses to water deficit (Source: Bohnert et al. 1999)

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1.9 Resurrection plants

Resurrection plants are angiosperms that possess the unique ability to withstand desiccation of their vegetative tissues and to revive from air dry state (Gaff 1987). These poikilohydrous plants can experience different rates of desiccation depending upon the water status of the environment and can recover uninjured from complete dryness within 80 hours (Sherwin & Farrant 1998, Mundree et al. 2002). Resurrection plants are widely distributed in all continents except Antarctica. According to Gaff (1987), they are mainly concentrated in arid climates such as South Africa, eastern South America and Western Australia, while only a few species have been found in Europe in the Balkan Mountains (Stefanov et al. 1992, Alpert 2005, 2006). Generally, about 330 species of angiosperms have been found to survive desiccation (Gaff 1987, Porembski & Barthlott 2000) but no resurrection gymnosperms have been reported (Hartung et al. 1998). There are both monocotyledonous resurrection plants such as *X. viscosa* and *Sporobolus stapfianus* and dicotyledonous species such as *Myrothamnus flabellifolia, Craterostigma plantagineum* and *Chamaegigas intrepidus*.

Despite their broad geographical distribution, the ecological range for resurrection plants is narrow. Usually they are found in habitats subjected to lengthy periods of drought, where rainfall is extremely sporadic, particularly on rock outcrops below 2000 m in tropical and subtropical areas, and to a lesser extent in temperate zones (Porembski & Barthlott 2000). Consequently, resurrection plants have evolved structures and mechanisms to allow survival under extreme conditions. As a result, these plants are a rich source of unique genes or stress-inducible promoters that can be used to improve stress tolerance in susceptible crop plants (Ramajulu & Bartels 2002). Accordingly, several genes postulated to play a role in desiccation tolerance have been isolated from *X. viscosa* (Mundree & Farrant 2000, Mowla et al. 2002, Garwe et al. 2003, Govender 2006).

1.10 The *XvSap1* gene

The *XvSap1* gene isolated and characterised by Garwe et al. (2003), is one of the genes postulated to play a role in desiccation tolerance in *X. viscosa*. Analysis of the cDNA sequence indicated a highly hydrophobic protein with six transmembrane regions. The deduced amino acid sequence showed a 49% identity to WCOR413, a low temperature-regulated protein from wheat and a 53% identity with another cold-associated protein in rice. The protein also showed between 25% to 56% identity to WCOR413-like proteins from *Arabidopsis.*

The *XvSap1* gene is, therefore, postulated to code for an integral membrane protein which is expressed in response to drought stress. Further studies revealed that the *XvSap1* gene could be a G protein-coupled receptor associated with signal transduction in osmotic stress (Iyer et al. 2007). Furthermore, transgenic *A. thaliana* plants transformed with the *XvSap1* gene demonstrated improved drought tolerance (Garwe et al. 2006). The promoter that controls the expression of the *XvSap1* gene in *X. viscosa* is of interest to the present study.

1.11 Plant transformation by particle bombardment

Biolistics is one of the two major DNA delivery techniques that has gained wide application in plant transformation. Transformation and recovery of fertile transgenic plants via particle bombardment was first reported in the late 20th century (Gordon-Kamm et al. 1990). Biolistic method involves fixing a plasmid or linearised DNA containing gene of interest onto tungsten or gold particles (micro-carriers). The micro-carriers are delivered to host cells at high speed so as to penetrate the nucleus of the plant cell. However, due to problems associated with biolistics such as high copy numbers and inability to transfer large DNA segments, this technology is losing its popularity paving the way for the most recent and promising

technology of *Agrobacterium*-mediated transformation.

1.12 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation uses a bacterial plasmid as a vector (Ishida et al. 1996, Frame et al. 2002). It results in a greater proportion of stable, low copy number transgenic events than does the biolistic gun (Ishida et al. 1996, Zhao et al. 1998), offers the possibility of transferring larger DNA segments into recipient cells (Hamilton et al. 1996), is highly efficient and possesses broad host range.

To harness *Agrobacterium* as a transgene vector, scientists have removed the tumor-inducing section of T-DNA, while retaining the T-DNA border regions and the *vir* genes. During transformation, a modified T-DNA vector is constructed in which the desired DNA fragment is inserted between the T-DNA border regions of *Agrobacterium*. This vector is transferred into *Agrobacterium* and virulence gene products actively recognise, excise, transport and integrate the T-DNA region into the host plant genome (Hooykaas & Schilperoort 1992). Using selectable markers, putative transformants are usually selected under stringent tissue culture conditions.

1.13 Plant tissue culture

Modern plant biotechnology has provided novel means for crop improvement through the integration and expression of defined foreign genes in plant cells, which can then be grown in vitro to regenerate whole plants. However, a basic prerequisite for the production of genetically transformed plants is efficient regeneration protocol that generates normal and fertile plants from single cells (Oduor et al. 2006).

The first somatic embryos in maize tissue culture were produced by Green & Phillips (1975). Reports of fertile maize plants regenerated from protoplasts (Shillito et al. 1989) were closely followed by the production of transgenic, fertile maize from

transformed suspension cell cultures of the hybrid A188 X B73 line (Gordon-Kamm et al. 1990). Maize cell lines derived from transformation competent sources such as immature embryos are heterogeneous for cells with differing embryogenic potential (Che et al. 2006).

Induction of embryogenic callus is genotype-specific in many plant species, including maize. Most maize elite lines remain inaccessible to improvement using standard transformation techniques either because of failure to produce embryogenic callus from transformation competent tissues, or inability to regenerate efficiently after embryogenic callus induction. Friable (Type II) callus (Armstrong & Green 1985) was found to be highly embryogenic and readily produced plants.

Previously, regeneration of gramineous species proved to be rather difficult because of their extreme recalcitrance to tissue culture manipulations *in vitro* (Zhang et al. 2002). The regeneration ability of any plant is influenced by different factors. The type of explant is considered one of the main factors that has attracted the concern of many investigators (El-Itriby et al. 2003). In addition, genetic background or the genotype of the explants has also been found to influence regeneration potentiality. Reports indicating the effect of the genotype and the role of nuclear genes in controlling the initiation of somatic embryos and regeneration in maize have been documented (Tomes & Smith 1985, Hodges et al. 1986, Willman et al. 1989, Bohorova et al. 1995). Moreover, regenerability is influenced, to a great extent, by the media composition (Armstrong & Green 1985, Vain et al. 1989a,b, Songstad et al. 1991, Bohorova et al. 1995, Carvalho et al. 1997).

Immature embryos have been the most widely used explant in many cereals, including maize (Green & Phillips 1975, Armstrong & Green 1985, Hodges et al. 1986, Shillito et al. 1989, Frame et al. 2002, El-Itriby et al. 2003, Oduor et al. 2006). Successful plant regeneration has also been reported from calli initiated from anthers (Ting et al. 1981), glumes (Suprasanna et al. 1986), immature inflorescences (Pareddy & Petolino 1990), immature tassels (Songstad et al. 1992, Rhodes et al.

1986), leaf segments (Ray & Ghosh 1990, Conger et al. 1987), seedling segments (Santos et al. 1984), shoot tips (O'Connor-Sanchez et al. 2002, Zhong et al. 1996), shoot apical meristems (Zhang et al. 2002) and mature embryos (Wang 1987, Rueb et al. 1994, Ozgen et al. 1998, Akula et al. 1999, Ward & Jordan 2001, Huang & Wei 2004).

1.14 Somaclonal variation

When regenerated plants are potted from cultures and grown up under standardised environmental conditions, the regenerants can exhibit transient, non-genetic or epigenetic changes as well as heritable, genetic variation (Karp 1995). Epigenetic changes appear to be a direct effect of the culture process, being physiological in origin and perhaps resulting from effects of the growth regulators in the medium (Fluminhan et al. 1996). Such epigenetic changes are of no value for crop improvement, as they are not expressed in sexual progeny.

However, studies have shown that changes in chromosome number and structure can occur in plants regenerated from tissue culture and that chromosome instability can be induced by media components, culture age, explant tissue and even by plant genotype (Peschke & Phillips 1992, Jain 2001). Chromosome variation in regenerable maize callus cultures have been investigated in few studies and only changes in chromosome number have been reported (Fluminhan et al. 1996).

The fact that somaclonal variation can occur in high frequency in regenerants, and that heritable single gene changes can occur in nuclear and organelle genomes, is potentially attractive to breeders. The simplest approach is to introduce the best available varieties into culture and then select for those somaclones that display incremental improvements as a result of somaclonal variation. The aim would not only be to retain all the favourable qualities of the variety, but also to add an additional trait such as drought tolerance, disease resistance or herbicide tolerance (Lindsey & Jones 1998).

1.15 Motivation and aims of the study

Plant genetic engineering has contributed substantially to the understanding of gene regulation and plant development in the generation of transgenic organisms for widespread usage in agriculture. As the application of genetically engineered plants has widened, so has the need to develop methods to fine-tune control of transgene expression. The availability of broad spectrum promoters that differ in the ability to regulate the temporal and spatial expression patterns of the transgene can significantly increase the successful application of transgenic technology (Potenza et al. 2004).

Currently, majority of genetically modified plants against various abiotic stresses have their transgenes expressed constitutively (Holmberg & Bulow 1998, Frame et al. 2000, 2002, Garwe et al. 2006). This poses the risk of overproduction of such proteins, which usually hamper the normal growth of plants and may result in dwarfism (Su et al. 1998, Lee et al. 2003). Therefore, successful genetic modification of crops against drought stress does not only require the skillful transfer of drought tolerant genes but also that the expression of such transgenes be controlled by stress-inducible promoters (Su et al. 1998, Kasuga et al. 1999, Lee et al. 2003, Schunmann et al. 2004).

The objectives of the present study were four-fold. The first facet aimed at determining the novelty of the full length XvPsap1 promoter sequence, predicting the regulatory elements present in the XvPsap1 promoter as well as developing single gene constructs driven by either full length promoter (XvPsap1) or truncated fragments (XvPsap2 or XvPsap3).

However, such computational predictions of promoter activity need to be validated by transformation of the XvPsap promoter fragments into various plant systems in order to verify the actual differential activity of the promoter in biological systems. As a result, the second aspect of this study focused on determining the most active

promoter fragment in a model transgenic monocot system, namely, Black Mexican Sweetcorn (BMS) cells.

To assess the potential application of XvPsap promoters in improving dicots against drought stress, the third aspect of the present study was to determine whether the XvPsap promoter fragments are functional in *N. tabacum* as well as determining the most active promoter fragment.

Since the present study was part of the larger project that seeks to genetically engineer maize for drought tolerance, the fourth aspect of this study aimed at assessing the activity of the most active promoter fragment in BMS and tobacco in whole maize.

Chapter 2

Bioinformatic analyses of the XvPsap1 promoter and development of gene constructs for genetic transformation

2.1 Summary

A non-coding sequence, XvPsap1 (2083 bp) isolated from the resurrection plant, *X. viscosa*, was screened *in silico* for similarity with existing plant promoters. No significant similarity with any known plant promoter was identified. The PlantCARE computational tool (Lescot et al. 2002) predicted various *cis*-acting elements including, but not limited to, those involved in drought stress, heat stress, abscisic acid, jasmonic acid, light and low temperature responsiveness. In addition, other regulatory elements commonly found in both promoter and enhancer regions were identified. These included meristem-specific activation, endosperm expression, auxin-responsive and circadian control elements. Two 5′ promoter deletions were generated resulting in truncated promoter fragments designated XvPsap2 (1577 bp) and XvPsap3 (1127 bp) relative to the translation start site. The full length promoter (XvPsap1) and truncated fragments (XvPsap2 and XvPsap3) were each cloned upstream of either the luciferase (*luc*) or green florescent protein (*gfp*) re-

porter genes. The nopaline synthase (*nos*) terminator was inserted downstream of each of the reporter genes. Subsequently, the promoter::luc/gfp::nos cassette was cloned into either the binary expression vector, pTF101.1 or the pA53 plasmid. A total of nine gene constructs were synthesised.

2.2 Introduction

Plant biotechnology expanded rapidly in the 1980s following the production of novel chimeric genes (Bevan et al. 1983, Fraley et al. 1983, Herrera-Estrella et al. 1983), transformation vectors (Hoekema et al. 1983, Bevan 1984), DNA delivery systems (Hernalsteens et al. 1980, Draper et al. 1982, Krens et al. 1982, Fromm et al. 1985, Sanford et al. 1987), combined with plant regeneration systems (Zambryski et al. 1983, Paszkowski et al. 1984, Shimamoto et al. 1989, Gordon-Kamm et al. 1990). This progress has made it possible to improve organisms with respect to agronomic or industrial traits through genetic engineering (Kishore & Shewmaker 1999). Numerous studies are now focussed on developing transgenic crops tolerant to abiotic stress (Garwe et al. 2006, Cong et al. 2008, Lal et al. 2008, Singla-Pareek et al. 2008, Takumi et al. 2008).

Sustainable development of transgenic stress-tolerant plants requires that the introduced gene(s) be regulated by a stress-inducible promoter to minimize undesirable phenotypes due to over expression (Su et al. 1998).

Previously, Garwe et al. (2003) isolated and characterised the *XvSap1* gene from *X. viscosa*. The gene was identified to confer tolerance to dehydration, high temperatures and salinity in model plants (Garwe et al. 2006). Recently, Iyer et al. (2007) contended that the *XvSap1* gene could be a G protein-coupled receptor associated with signal transduction in osmotic stress. In order to identify the promoter that naturally regulates the expression of the *XvSap1* gene in *X. viscosa*, a 2083 bp sequence, designated XvPsap1, was isolated upstream of the gene (Ingle, pers. comm.).

The bioinformatic analyses of the XvPsap1 promoter was undertaken in this study. To determine the shortest functional length of the promoter, 5′ promoter deletion analyses was performed, resulting in two truncated promoter fragments designated XvPsap2 (1577 bp) and XvPsap3 (1127 bp) relative to the translation start site. The subsequent cloning of these promoter fragments upstream of either the *luc* or *gfp* reporter gene is described. The cloning of the *nos* terminator downstream of each of the reporter genes as well as the cloning of the promoter::luc/gfp::nos expression cassettes into either the pA53 or the binary expression vector, pTF101.1 is also described.

The objectives were three-fold: (i) to verify the novelty of the full length XvPsap1 promoter sequence using comparative genomics; (ii) to predict the regulatory elements present in the XvPsap1 promoter using genome browers; and (iii) to develop single gene constructs driven by either XvPsap1, XvPsap2 or XvPsap3.

2.3 Materials and methods

2.3.1 Bionformatic analyses of the XvPsap1 promoter

Following sequencing, the level of sequence homology between XvPsap1 and existing plant promoters was assessed by searching the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm (Altschul et al. 1990). Thereafter, the promoter sequence was analyzed for the presence of regulatory elements using the PlantCARE software (Lescot et al. 2002).

2.3.2 Cloning of XvPsap1, XvPsap2 and XvPsap3 into pTF101.1

The XvPsap1 promoter, which had been previously cloned into pBluescript was amplified using a forward primer designated SAP-prom-EcoRI-F_1 (Appendix Table B.5) and a reverse primer designated SAP-prom-BamHI-R_2 (Appendix Table B.5). The reverse primer was used in the amplification of all three promoter fragments as they varied only in the 5′ ends. To amplify XvPsap2 and Xvpsap3, forward primers designated SAP-prom-EcoRI-F_2 and SAP-prom-EcoRI-F_3 (Appendix Table B.5) were used, respectively.

The amplification was carried out with the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s; 58°C for 30 s, and 72°C for 90 s; and a final extension step of 72°C for 5 min. The PCR reaction was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Singapore). For each amplification, 25 μl reaction volumes were set up with component concentrations as described in Appendix Table B.1. The *Taq* DNA polymerase, PCR buffer and $MgCl_2$ were supplied by New England Biolabs (USA).

Amplimers generated by PCR were electrophoresed on a 1% agarose gel. The amplimers were thereafter purified using the EZ-10 Spin Column PCR product purification Kit (Bio Basic, Canada) according to the manufacturer's instructions

(Appendix A.1). The purified amplimers were digested using *Eco*RI and *Bam*HI in a 20 μl reaction volume (Appendix Table B.2) and incubated overnight at 37°C (Sambrook & Russell 2001). Alternatively, and following the manufacturer's instructions (Appendix Table B.4), the amplimers were initially cloned into pDrive (Qiagen, USA; Appendix Fig. B.2) prior to performing the restriction digest.

The digested amplimers were electrophoresed on a 1% agarose gel. The DNA fragments of the correct size were excised and purified using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada) according to the manufacturer's instructions (Appendix A.2). Similarly, pTF101.1 (Appendix Fig. B.1) was digested with *Eco*RI and *Bam*HI, electrophoresed, excised and purified.

The purified digested amplimers and linearised pTF101.1 were ligated using standard ligation conditions (Appendix Table B.3). The T4 DNA ligase and 10X ligation buffer were supplied by NEB (USA). The reaction volume was made up to 10 μl, mixed well and incubated overnight at 4°C. The individual ligation mixtures of recombinant pTF101.1 containing either XvPsap1, XvPsap2 or XvPsap3 were transformed into competent *E. coli* cells.

2.3.3 Transformation of recombinant pTF101.1::XvPsap into competent *E. coli*

The recombinant pTF101.1 plasmids (pTF101.1::XvPsap1, pTF101.1::XvPsap2, and pTF101.1::XvPsap3) were transformed into competent *E. coli* cells. The competent cells were allowed to thaw on ice. Ten microlitres of ligation mix was added to the competent cells and mixed gently. The transformation mix was incubated for 10 min on ice. The cells were thereafter heat shocked by incubation for 5 min at 37°C followed immediately by incubation for 2 min on ice. Eight hundred microlitres of LB broth (Appendix Table A.7) was added to the transformed cells and incubated for 50 min at 37°C with vigorous shaking. Fifty microlitres of the transformation mix was plated onto LB agar (Appendix Table A.7) plates

supplemented with spectinomycin (100 μg/ml) and incubated overnight at 37°C.

Colony PCR was performed to screen for positive clones using promoter specific primers (SAP-prom-BamHI-R_2, SAP-prom-EcoRI-F_1, SAP-prom-EcoRI-F_2 and SAP-prom-EcoRI-F_3). The nucleotide sequence of the cloned XvPsap promoter fragments in pTF101.1 were confirmed by sequencing. Colonies confirmed by sequencing to contain the correct recombinant plasmids were treated with glycerol and stored at −80°C (Appendix A.4).

2.3.4 Cloning of the *nos* gene into pDrive

The *nos* terminator (300 bp; Appendix Fig. B.8) was amplified using gene specific primers nos-speI XF2 and nos-hindIII XR1 (Appendix Table B.5). The amplification was carried out with the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s; 57°C for 30 s, and 72°C for 60 s; and a final extension step of 72°C for 5 min. The *nos* amplimer was purified and ligated into pDrive (standard protocol, see section 2.3.2).

The recombinant pDrive plasmid (pDrive::nos) was transformed into competent *E. coli* cells (standard protocol, see section 2.3.3). The transformed cells were plated on LB agar supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), X-gal and 0.2 mM of isopropyl-β-D-thiogalactoside. Colony PCR was performed on white colonies using specific primers (nos-speI XF2 and nos-hindIII XR1).

2.3.5 Cloning of the *luc* gene into pDrive

The firefly luciferase gene (1656 bp; Appendix Fig. B.7) was amplified from pBluescript using gene specific primers (Luc-BamHI F and Luc-SpeI R2). The PCR parameters and subsequent cloning into pDrive were according to standard protocols (see sections 2.3.2 and 2.3.4).

2.3.6 Generation of luc::nos constructs

The recombinant plasmids, pDrive::luc and pDrive:nos, were isolated from cells that had been cultured overnight at 37°C in LB broth supplemented with ampicillin (100 μg/ml). Plasmid DNA extraction was performed using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic, Canada) according to the manufacturer's instructions (Appendix A.3). The pDrive::luc plasmid was thereafter restricted with *Bam*HI and *Spe*I to release the *luc* gene (standard protocol, see section 2.3.2). Similarly, the pDrive::nos plasmid was linearised following digestion with the same enzymes. The *luc* digestion product and linearised pDrive::nos plasmid were ligated (standard protocol, see section B.3). The recombinant pDrive::luc::nos plasmid was transformed into competent *E. coli* cells (standard protocol, see section 2.3.3) and positive colonies were identified.

2.3.7 Cloning of luc::nos constructs into pTF101.1 binary vector containing the XvPsap1, XvPsap2 and XvPsap3 promoter fragments

Recombinant pTF101.1 harbouring the promoter fragments were independently digested with *Bam*HI and *Hin*dIII. Similarly, pDrive::luc::nos was digested with the same enzymes to release the luc::nos construct. The linearised pTF101.1 and luc::nos constructs were ligated and thereafter transformed into competent *E. coli* cells (standard protocol, see section 2.3.3). The transformed cells were plated onto LB agar supplemented with spectinomycin (100 μg/ml). Colony PCR was performed on white colonies using gene specific primers.

2.3.8 Transformation of *A. tumefaciens* with pTF101.1 containing XvPsap promoters expressing the *luc* gene

The three pTF101.1 recombinant plasmids containing XvPsap1::luc::nos, XvPsap2::luc::nos and XvPsap3::luc::nos were isolated from *E. coli* and transformed

into competent *A. tumefaciens*. The transformed *A. tumefaciens* cells were incubated at 30°C instead of 37°C. In addition, selection was performed using spectinomycin (100 μg/ml) and kanamycin (50 μg/ml).

Positive transformants were screened initially by colony PCR using *luc* specific primers. Glycerol stocks were synthesised and stored at −80°C.

2.3.9 Cloning of expression cassettes into pA53

Recombinant pTF101.1 was digested with *Eco*RI and *Hin*dIII to release either XvPsap1::luc::nos, XvPsap2::luc::nos or XvPsap::luc::nos. Similarly, the pA53 plasmid (Appendix Fig. B.3) was digested with *Eco*RI and *Hin*dIII to linearise the vector. The inserts were individually ligated into pA53 (standard protocol, see section 2.3.2) generating three separate pA53 constructs.

2.3.10 Cloning of the *gfp* gene into pA53

The *gfp* gene (759 bp; Appendix Fig. B.9) was amplified using gene specific primers, GFP-BamHI F and GFP-speI R (Appendix Table B.5). The amplification was performed under the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s; 56°C for 60 s, and 72°C for 60 s; and a final extension step of 72°C for 5 min. The *gfp* amplimer was cloned into pDrive. The pDrive::gfp plasmid was digested with *Bam*HI and *Spe*I to release the *gfp*, which was ligated into pA53 to replace the *luc* gene in the XvPsap1::luc::nos, XvPsap2::luc::nos and XvPsap3::luc::nos constructs.

2.4 Results

2.4.1 Bioiformatic analyses of XvPsap1 for regulatory motifs

Bioinformatic analyses of the XvPsap1 sequence were performed. No significant identity was observed to any known plant promoter. Various putative *cis*-acting elements were identified including those involved in dehydration, heat stress, abscisic acid, jasmonic acid, light and low temperature responsiveness. In addition, common *cis*-acting elements involved in meristem specific activation, endosperm expression, auxin-responsive and circadian control were also identified. The major regulatory motifs identified in the promoter are listed in Table 2.1. Full information on host organism, position, strand, matrix score and motif sequence of the promoter regulatory elements as predicted by the plantCARE is described in Appendix Table C.1.

Table 2.1: A summary of regulatory elements identified in XvPsap1 promoter using PlantCARE software (Lescot et al. 2002)

Site name	Function
ABRE	*cis*-acting element involved in abscisic acid responsiveness
MBS	MYB binding site involved in drought-inducibility
HSE	*cis*-acting element involved in heat stress responsiveness
G-Box	*cis*-acting element involved in light responsiveness
Box I	*cis*-acting element involved light responsiveness
CAAT-box	common *cis*-acting element in promoter and enhancer regions
circadian	*cis*-acting element involved in circadian control
CCGTCC-box	*cis*-acting element related to meristem specific activation
CGTCA-motif	*cis*-acting element involved in MeJA-responsiveness
LTR	*cis*-acting element involved in low-temperature responsiveness
Skn-1 motif	*cis*-acting element required for endosperm expression
TC-rich repeats	*cis*-acting element involved in defense and stress responsiveness
TGA-box	part of an auxin-responsive element
TCA-element	*cis*-acting element involved in salicylic acid responsiveness
TATA-box	core promoter element around -30 of transcription start

2.4.2 Recombinant pA53 constructs for particle bombardment

Sequencing results confirmed that the cloning of XvPsap1, XvPsap2 and XvPsap3 promoter fragments expressing either *luc* or *gfp* in pA53 was successful. A total of six gene constructs were developed. The first set of gene constructs contained the three different promoter sizes driving the expression of *luc* (Fig. 2.1 A1, A2, A3). The second set of gene constructs, harboured the three different promoter sizes regulating the expression of *gfp* (Fig. 2.1 B1, B2, B3).

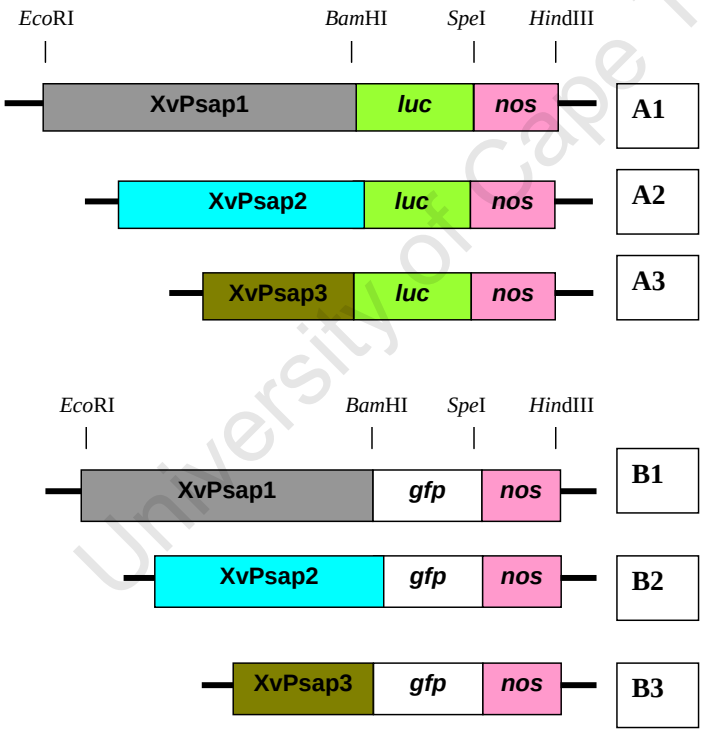

Figure 2.1: Schematic illustration of recombinant pA53 expression cassettes used in biolistic gene delivery. *nos*, nopaline synthase gene. **A1**, **A2**, and **A3**, pA53 gene constructs expressing *luc*. **B1**, **B2**, and **B3**, pA53 gene constructs expressing *gfp*.

2.4.3 Recombinant pTF101.1 constructs for *Agrobacterium tumefaciens*-mediated transformation

The synthesis of XvPsap1, XvPsap2 and XvPsap3 promoter fragments expressing *luc* in pTF101.1 was successful (Fig. 2.2). A total of three constructs were generated. Sequencing confirmed the presence of the target sequences.

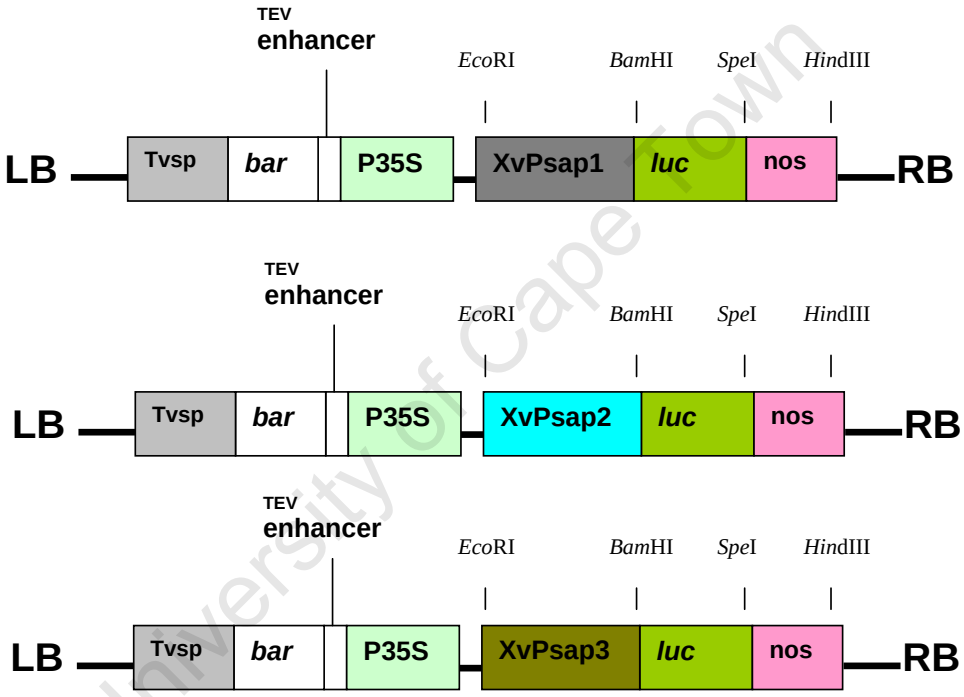

Figure 2.2: Schematic illustration of the T-DNA region of pTF101.1 containing varying promoter fragments expressing *luc*. **LB**, Left border; **Tvsp**, soybean vegetative storage protein terminator; *bar*, phosphinothricin acetyltransferase gene; **P35S**, CaMV 35S promoter; XvPsap1, XvPsap2, XvPsap3, truncated fragments of the XvPsap promoter; *luc*, luciferase gene; *nos*, nopaline terminator; **RB**, right border.

2.5 Discussion

The accumulation of plant biological databases, specifically on plant promoter and transcription factors has furthered the understanding of the function of genomes (Yamamoto et al. 2007). These databases can be utilised for more accurate comparisons and analyses of plant regulatory elements (Venter & Botha 2004). Of these databases, the BLAST (Altschul et al. 1990) is the most widely used and well-established alignment search tool compared to other search systems like ClustalW and ClustalX (Aiyar 2000).

In this study, *in silico* screening of XvPsap1 using the BLAST tool revealed that the XvPsap1 sequence had no significant homology to any known promoter sequence. These results clearly demonstrate that the XvPsap1 sequence is novel. This fact added to other features make XvPsap1 suitable for commercial exploitation. The XvPsap1 sequence has consequently been patented (Patent File Ref. PA143860/PCT).

Bioinformatic analyses of the XvPsap1 sequence was undertaken as it would allow predictions regarding promoter positions and expression profiles as well as its hidden transcriptional networks (Venter & Botha 2004). Other studies have identified numerous functional elements as promoter constituents for precise and regulated transcriptional initiation (Carey & Smale 2001, Butler & Kadonaga 2002, Smale & Kadonaga 2003). Of the many computational databases that exist, Hehl & Wingender (2001) contend that PlantCARE (Rombauts et al. 1999, Lescot et al. 2002), which is widely used in plant promoter analyses, is the most suited for the identification of TF-binding sites and *cis*-acting elements. Using PlantCARE software, the presence of several regulatory motifs such as ABRE, MBS and HSE were putatively identified in XvPsap1. These results strongly suggest that the XvPsap1 promoter may be involved in the abiotic stress response. Given that the XvPsap1 promoter was isolated 2083 bp upstream of the *XvSap1* gene, which Garwe et al. (2006) reported to confer stress tolerance to *A. thaliana* under salinity, osmotic

and high temperature stress, these results strongly support the conclusion that the XvPsap1 sequence could contain the *cis*-acting elements, which naturally regulate the expression of *XvSap1* gene in *X. viscosa*.

The presence of major regulatory motifs does not only point to the XvPsap1 sequence being a promoter, but also reveals that it is stress-inducible. Albeit further research needs to be conducted on XvPsap1 regarding its activity in biosystems, these preliminary results emphasise the potential utility of this promoter with respect to improving crops such as maize for drought tolerance. Although these computational genome browsers can be very useful, they do not provide definitive answers to every question. Hence, the presence of regulatory elements does not necessarily guarantee promoter activity. It is therefore desirable that such computational predictions be accompanied by transformation of the XvPsap1 promoter into various plant systems in order to verify the actual activity of the promoter.

When developing transgenic plants, the use of minimum trans-sequence is desirable. This is because changes in trans-sequence organization such as truncation, inversion, deletion and other complex rearrangements have been reported to increase when long trans-sequences are used (Jorgensen et al. 1996). Trans-sequence rearrangements are particularly common among transgenic plants derived from biolistic delivery of DNA (Dai et al. 2001). Physical force used to deliver DNA into cells may contribute to these rearrangements (Bhat & Srinivasan 2002). Furthermore, long trans-sequences coupled with plasmid vector DNA sequences, which in many instances also get transferred, compromise the transformation efficiency. This has been documented even for *Agrobacterium*-mediated transformation where a more precise mechanism operates in the transfer of T-DNA (Iglesias et al. 1997, Kononov et al. 1997, De Buck et al. 2000).

It was therefore an important objective of this study to determine the shortest functional length of the XvPsap1 promoter to minimise the use of long trans-sequences. Experimental characterisation of regulatory promoter elements are nor-

mally conducted by sequential deletion of promoter fragments and promoter, with gain-of-function *cis*-element, activities is assessed on transient and stable level in transgenic plants (Koch et al. 2001). A similar approach was undertaken in this study.

Success in genetic engineering requires careful cloning of appropriate expression cassettes involving promoter regions, target genes and the terminator sequences all in conducive expression vectors (Conner & Jacob 1999). This study, demonstrates successful cloning of single gene constructs that would find application in genetic engineering either via particle bombardment or *Agrobacterium*-mediated transformation. Accordingly, transformation of Black Mexican Sweetcorn (BMS) cells through particle bombardment is reported in Chapter 3.

Chapter 3

Functional analysis of XvPsap promoter activity in BMS cells

3.1 Summary

Gene constructs containing XvPsap1 and its truncated fragments driving the expression of either *luc* or *gfp* were used in the transformation of BMS cells by particle bombardment. Southern blot analysis was performed to determine stable introgression of the expression cassette into the genomes of the BMS cells. The functional properties of each promoter fragment under salt stress treatment were examined by fluorescence quantitative analyses. Cells transformed with XvPsap1::luc, XvPsap2::luc and XvPsap3::luc displayed increased luciferase activity. However, cells containing XvPsap1::luc displayed the highest luciferase activity while those containing XvPsap3::luc displayed the lowest. The qRTPCR analysis demonstrated that the *luc* gene was upregulated within 24 h of salt stress in both transgenic cell lines containing either XvPsap1 (5-fold) or XvPsap2 (1.9-fold). In contrast, the XvPsap3 promoter demonstrated mild activity (1.9-fold) only after 48 h of stress. These results suggest that the longest promoter fragment (XvPsap1, 2083 bp) is the most active in BMS cells.

3.2 Introduction

Plant transformation is an important tool in the hands of molecular biologists (Bhat & Srinivasan 2002). One method of DNA delivery, particle bombardment, has been widely used to transform recalcitrant species such as cereals (Vain et al. 1995). For example, Shepherd et al. (2007) recently reported the development of maize streak virus resistant transgenic maize generated by particle bombardment. Particle bombardment accounted for 25% of all the plant transformations reported up to 2003 (Vain 2007).

Plant cell cultures are useful model systems to investigate the function and regulation of genes (Hano et al. 2008). For instance, *in vivo* characterisation of plant promoters in transgenic maize suspension cells has been reported (Tuerck & Fromm 1994). This is due to the ability to control growth conditions of cell suspensions as well as the unlimited supply of undifferentiated cells amenable to genetic manipulations (Rasmussen et al. 1994).

When using suspension cells for genetic transformation, the viability of the target cell lines is usually determined using chemosensitivity assays such as MTT (Plumb et al. 1989). The MTT assay is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan producing a purple colour. This mainly occurs in mitochondria, and as such it is in large, a measure of mitochondrial activity (Mosmann 1983, Vistica et al. 1991, Wilson 2000).

Particle bombardment of BMS cells using *gfp* and *luc* gene constructs regulated by varying lengths of the XvPsap promoter is reported. Functional analysis of promoter activity is described. The primary objective was to determine the most active promoter fragment in a model monocot system.

3.3 Materials and Methods

3.3.1 BMS cell culture and growth conditions

Cell cultures were grown on BMS culture media in the dark at room temperature. The BMS cells were subcultured onto fresh media every 2 weeks. The BMS culture media was composed of MS (Murashige & Skoog 1962) salts and vitamins supplemented with 2,4-D (1 mg/l), myo-inositol (100 mg/l), sucrose (30 mg/l) and agar (8 g/l). The pH of the media was adjusted to 5.8. Suspension cells were cultured in liquid BMS media (excluding agar) at 27°C in a shaking incubator (MRC, Israel).

3.3.2 MTT assay

Aliquots of cells were collected every 24 h over a 7 day period to determine viability. The 180 μl aliquot of cells was transferred to a sterile 2 ml Eppendorf tube containing 20 μl of MTT. The mixture was vortexed for 30 min at room temperature and thereafter centrifuged for 5 min at 12,000*g*. The supernatant was transferred to a fresh tube and 1 ml of DMSO added. The vortexing and centrifugation step was repeated as described above. Thereafter, 200 μl of cell-free supernatant was transferred to a 96 well plate and spectrophotometric readings taken at 570 nm using a microplate reader installed with KC4 software (Bio-Tek instruments, USA).

3.3.3 Transformation of BMS cells

Prior to co-bombardment, BMS cells were subcultured overnight on high osmotic BMS media (BMS media supplemented with 18 g/l mannitol). One microgram each of the construct of interest and of pAHC25, containing the *bar* gene for herbicide selection, were precipitated onto 1 μM gold particles (50 μl of 60 mg/ml gold suspended in 50% glycerol) (Dunder et al. 1995). Transformation was conducted using a PDS-1000-He Biolistic Bombardment Delivery System (Bio-Rad Laboratories, Germany) with a pressure of 650 psi and a gap distance of 6 mm between

the rupture disc and macrocarrier containing the DNA. Each sample was bombarded twice. A non-bombarded control plate was included in all experiments. The BMS cells transformed with the *luc* containing constructs were initially selected on BMS media harbouring bialaphos (3 mg/l) prior to imposing salt treatment. Only bialaphos resistant cells were assessed for the presence of *bar* and *luc* transgenes.

3.3.4 Detection of transgenic BMS cells

Genomic DNA was extracted using the CTAB extraction method (Stacey & Isaac 1994, Dehestani & Tabar 2007) with modifications. Instead of grinding cells in liquid nitrogen using a mortar and pestle, warm CTAB extraction buffer was added to 300 mg BMS cells in 2 ml Eppendorf tubes. Two $\frac{1}{4}$ inch ceramic spherical beads (Bio 101, USA) were added and the cells homogenized by vortexing the mixture for 10 min.

The presence of the *bar* gene was determined by amplification of a 421 bp fragment of the gene using the primer pair Bar I and Bar II (Appendix Table B.5) with the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s; 57°C for 30 s, and 72°C for 90 s; and a final extension step of 72°C for 5 min. Similarly, to determine the presence of the *luc* gene, the primer pair Luc F and Luc-SpeI R2 was used to amplify a 1.2 kb fragment of the gene. The amplification was carried out with the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s; 58°C for 30 s, and 72°C for 90 s; and a final extension step of 72°C for 5 min.

3.3.5 Southern blot analysis of transgenic BMS cells

For Southern blot analysis, 10 μg of genomic DNA from each transgenic BMS cell line was digested overnight with *Spe*I and *Bam*HI at 37°C. The digestion products were electrophoresed overnight at 40 V on a 1% agarose gel. The separated DNA was blotted onto a nylon membrane (Hybond N^+; Amersham Biosciences, USA) by capillary transfer (Sambrook et al. 1989). The transferred DNA was fixed using

a Hoefer UVC 500 crosslinker (Amersham Biosciences, USA). A 1.2 kb fragment of the *luc* gene was labeled with digoxigenin (DIG) using the PCR Labeling Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Hybridisation and detection of probe was carried out using a non-radioactive, DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics, Germany) according to the manufacturer's instructions.

The *luc* gene was amplified using the primer pair, Luc F and Luc-SpeI R2 (see section 3.3.4). The generated product was electrophoresed on a 1% agarose gel and a 1.2 kb band was excised and purified (see section 2.3.2). The purified amplimer was used as template for probe synthesis using a DIG-dUTP:dTTP ratio of 1:6. Reactions were performed on a Gene Amp 9700 thermocycler with the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s; 58°C for 30 s, and 72°C for 90 s; and a final extension step of 72°C for 5 min. Labeling efficiency was assessed by electrophoresing the labeled product on a 1% agarose gel. The tissue type plasminogen activator (tPA) was used as positive control.

Hybridisation with the labeled *luc* probe was performed for 16 h at 42°C. The blots were initially washed with 2X SSC buffer at room temperature and thereafter more stringently with 0.1X SSC buffer. The CDP-*star* chemiluminescent substrate (Roche Diagnostics, Germany) for alkaline phosphatase was used for detection according to the manufacturer's instructions.

3.3.6 Salt stress treatment

The BMS cells that showed the presence of *bar* and *luc* transgenes were further subcultured on selection media before being transferred to BMS media supplemented with either 200 mM NaCl or sorbitol. Aliquots (100 μl) of cells were collected every 24 h over a 3 day period. The BMS cells transformed with *gfp* constructs were viewed after 36 h of salt stress using an inverted phase contrast epifluorescence ELWD microscope (Nikon, Japan). The emitted fluorescence was imaged on

a computer using a filter to detect the fluorescence through a 20X magnification objective at wavelengths longer than 510 nm.

3.3.7 Analysis of luciferase activity in transgenic BMS cells

The activity of luciferase under salt treatment was monitored for 72 h using a Modulus Microplate Multimode Reader (Tuner Biosystem, USA). Prior to salt induction, samples were treated with a 1 mM beetle luciferin solution to eliminate any residual luciferase activity. Luciferase assay reagent was reconstituted by adding 10 ml luciferase assay buffer in a vial containing luciferase assay substrate and mixing thoroughly at room temperature.

The 1X lysate buffer provided with the Luciferase Assay Kit contains triton-X100 which compromises the output of the Braford reaction (Bradford 1976) and consequently was not used. Instead, an alternative extraction buffer (0.1 M potassium phosphate buffer pH 7; 1 mM dithiothreitol) was prepared. The BMS cell sample (200 mg) was transferred to a sterile 2 ml Eppendorf tube, ground in 0.5 ml cold extraction buffer and centrifuged for 5 min at 10,000g at 4°C (Ow et al. 1986). The supernatant was transferred to a sterile tube and incubated on ice. An aliquot (20 μl) of each extract was transferred to a 96 well plate and quantitative measurements taken. Luciferase activity was measured in terms of relative light units (RLU) following the injection of 100 μl of the assay reagent. Luciferase assays were performed in triplicate on each extract and the concentration of the extract determined using BSA.

3.3.8 RNA extraction from transgenic BMS cells

Total RNA was extracted using the TRIzol method (Chomczynski & Sacchi 1987) according to the manufacturer's instructions (Life Technologies, USA). An 200 mg aliquot of BMS cells was ground with liquid nitrogen using a mortar and pestle. The ground cells were mixed with 1 ml TRIzol reagent and thereafter incubated

for 5 min at room temperature. The mixture was centrifuged for 10 min at 12,000g at 4°C. The supernatant was transferred to a sterile 2 ml Eppendorf and 200 μl of chloroform was added. The tube was incubated for 2 min at room temperature, vortexed for 15 s and allowed to settle for 3 min at room temperature. Thereafter, the mixture was centrifuged for 15 min at 10,000g at 4°C and the RNA was precipitated from the upper phase by adding a half volume each of isopropanol and 0.8 M sodium citrate/1.2 M NaCl. The mixture was allowed to settle for 10 min at room temperature and thereafter pelleted by centrifugation for 10 min at 10,000g at 4°C . The pellet was washed with 70% EtOH, vortexed briefly and centrifuged again for 10 min at 10,000g at 4°C. The pellet was air dried for 5 min and dissolved in 89 μl of 0.01% DEPC treated water. Dissolution was enhanced by incubating the tube for 10 min at 55°C. The RNA sample was centrifuged for 5 min at 10,000g at room temperature and the insoluble pellet discarded. Ten microlitres of DNase I buffer (New England Biolabs, USA) was added to each 89 μl RNA sample and mixed gently. One microliter of DNase I was added to each sample to eliminate residual DNA followed by incubation for 10 min at 37°C. The sample was purified using the EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio Basic, Canada) according to the manufacturer's instructions. The purified RNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The RNA quality was further assessed by visualizing on a 1.2% agarose gel.

3.3.9 Synthesis of cDNA

One step cDNA synthesis was performed (Clontech, USA) according to the manufacturer's instruction. Purified RNA (2.5 μg) was reverse transcribed in a 25 μl reaction. The initial reaction comprised RNA, 0.5 μg of random hexamers and RNase-free water to 12.5 μl. The reaction contents were gently mixed and thereafter incubated for 2 min at 72°C. Following the initial incubation the samples was immediately chilled on ice. The reverse transcription master mix was prepared in a separate tube and comprised 100 U M-MuLV RNase H^- reverse transcriptase

(Finnzymes, Finland), 2.5 μl of 10X M-MuLV RT buffer, 2 μM dNTPs and RNase-free water to a volume of 12.5 μl per reaction. The enzyme mix was transferred to the individual RNA mixes and the reaction incubated for 1 h at 42°C. The RNA was thereafter degraded using 2.5 U RNaseH (New England Biolabs, USA) with sequential incubation for 5 min at 25°C followed by 15 min at 37°C. The cDNA pool was immediately chilled on ice and diluted 10-fold with DEPC-treated distilled H_2O and stored at −80°C.

3.3.10 Real-time quantitative PCR

The relative expression of *luc* was determined by qRTPCR using the SensiMix DNA Kit (Quantace, Australia). Aliquots of a single cDNA sample were used with all primer sets. Reactions were performed in a 25 μl volume containing 1 μl of each primer (10 μM), 2 μl cDNA, 0.5 μl 50X SYBR Green and 12.5 μl 2X SensiMix PCR master mix.

The primer pair qRTLuc F1 and qRTLuc R2 (Appendix Table B.5) specific for *luc* was used to amplify a 192 bp fragment of the gene. The 18S rRNA was used as reference gene and was detected using the primer pair qRT18S-F2 and qRT18S-R2 (Appendix B.5). The primers were designed using the Primer3 programme (Rozen & Skaletsky 2000). Real-time PCR was performed on a Rotor-Gene RG-3000A PCR machine (Corbett Research, Australia) in a 72-well reaction plate with the following cycling parameters: 95°C for 10 min; 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 5 s. Amplification specificity was verified at the end of the PCR run by analysing a melting curve of the amplimer generated. In addition, an aliquot of the amplimer was electrophored on a 2% agarose gel. Each qRTPCR reaction was performed in triplicate.

Serial dilutions of pooled cDNA from the treated samples were used to generate five-point standard curves. Reaction efficiencies were determined from the standard curves. Gene constructs containing the *luc* gene were used as templates for positive

controls.

The standard curve was imported into every run and used to calculate concentrations of the gene of interest (GOI) and the housekeeping gene (HKG). To normalize the run-to-run variations in PCR efficiencies, one of the standard solutions was included in every run and used to correct for variations in the run. The calculated concentration of the GOI was divided by the calculated concentration of the HKG. Thereafter, the values obtained from three biological replicates were averaged and used for relative quantification of transcripts. To evaluate the pattern of *luc* expression under stress conditions, the transcript levels in samples prior to the application of the stress (i.e. time zero) were used as a reference. The mean relative transcript level was determined and standard deviation values reported as n-fold relative to the 18S rRNA expression levels (Livak 1997, Brunner et al. 2004, Wong & Medrano 2005).

3.3.11 Promoter activity data analyses

Promoter activity data was analysed using GraphPad Prism (version 5.00; GraphPad Software, USA). The translation and expression levels of luciferase in transgenic BMS cells presented as n-fold relative to 0 h stress treatment were assessed for statistical differences. Statistically significant differences were determined using a one-way ANOVA (Kruskal-Wallis test) and by making pair-wise comparisons between least square means. Means were considered significantly different at a level of 0.05.

3.4 Results

3.4.1 Cell viability and salt treatment

The BMS cells were aliquoted every 24 h for the MTT assay to minimize variations due to circadian rhythms. After 4 days of culture, browning of the treated cell suspension was observed compared to the untreated control. The browning was accompanied by slow growth with cells treated with NaCl exhibiting a greater effect than those treated with sorbitol (Fig. 3.1 A). A significant survival inhibition (< 50% survival) was noted in cells treated with NaCl (Fig. 3.1 B) and was therefore selected for downstream stress treatments.

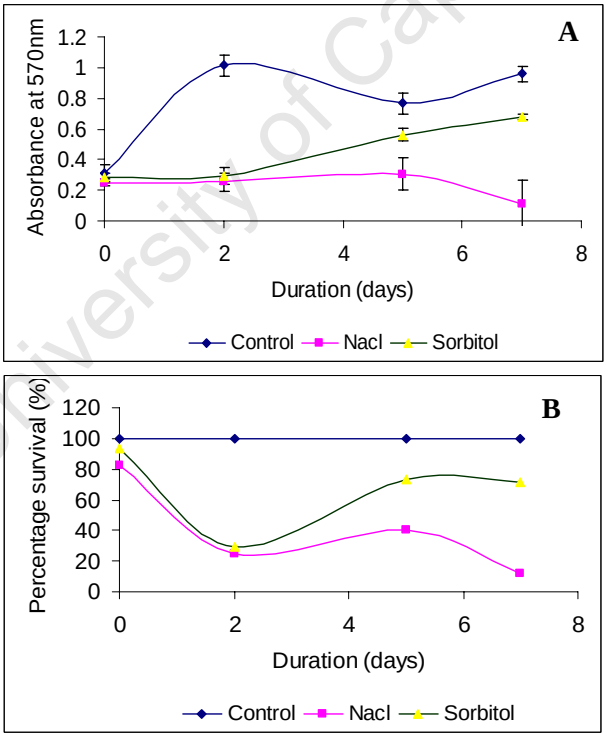

Figure 3.1: Graphical representation of the MTT assay of BMS cells under NaCl and sorbitol treatments. **A**: Growth of BMS cells treated with either 200 mM NaCl or sorbitol. **B**: Percentage survival of BMS cells under either 200 mM NaCl or sorbitol, calculated relative to untreated controls. The data are presented as means ± SEM from three independent transgenic BMS cells.

3.4.2 Detection of GFP fluorescence

Fluorescence was monitored in transgeninc BMS cells containing promoter::gfp constructs after 36 h of treatment with 200 mM NaCl. Non-transformed BMS cells did not display any GFP fluorescence (Fig. 3.2 A). However, fluorescence was apparent in cells bombarded with *gfp* under the control of a constitutive promoter (Fig. 3.2 B). Similarly, GFP fluorescence was observed in all cells transformed with the different promoter fragments (Fig. 3.2 C, D, E). Due to inability to quantify GFP fluorescence, only those constructs containing the *luc* gene were used for further analysis of promoter activity.

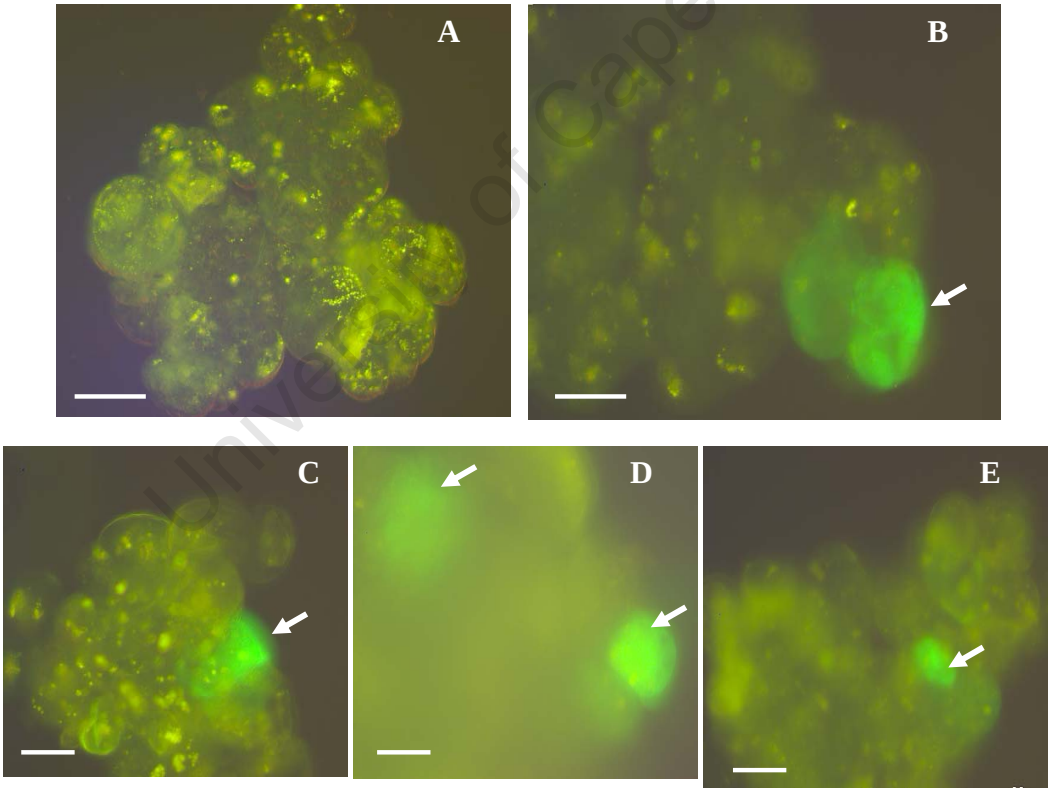

Figure 3.2: GFP expression in transgenic BMS cells following 36 h of treatment with 200 mM NaCl. **A**: Non-transformed BMS cells (control). **B**: BMS cells with constitutive GFP expression. **C**, **D**, and **E**: BMS cells transformed with the *gfp* gene under control of the XvPsap1, XvPsap2, and XvPsap3 promoter fragments, respectively. The green fluorescent sections of the calli are indicated by the arrows (scale bars = 0.2 mm).

3.4.3 Transformation and selection of transgenic BMS cells containing the *luc* gene

The BMS cells were co-bombarded with pA53 containing the different promoter fragments and pAHC25 containing the *bar* gene. Non-transformed cells displayed complete necrosis following 4 weeks of bialaphos selection (Fig. 3.3 A). However, these cells thrived on media lacking bialaphos (Fig. 3.3 B). The cells transformed with the *luc* gene under the control of XvPsap1, XvPsap2 and XvPsap3 promoters displayed partial necrosis (Fig. 3.3 C, D, E). Only those cells that displayed bialaphos resistance were subcultured and subjected to PCR screening.

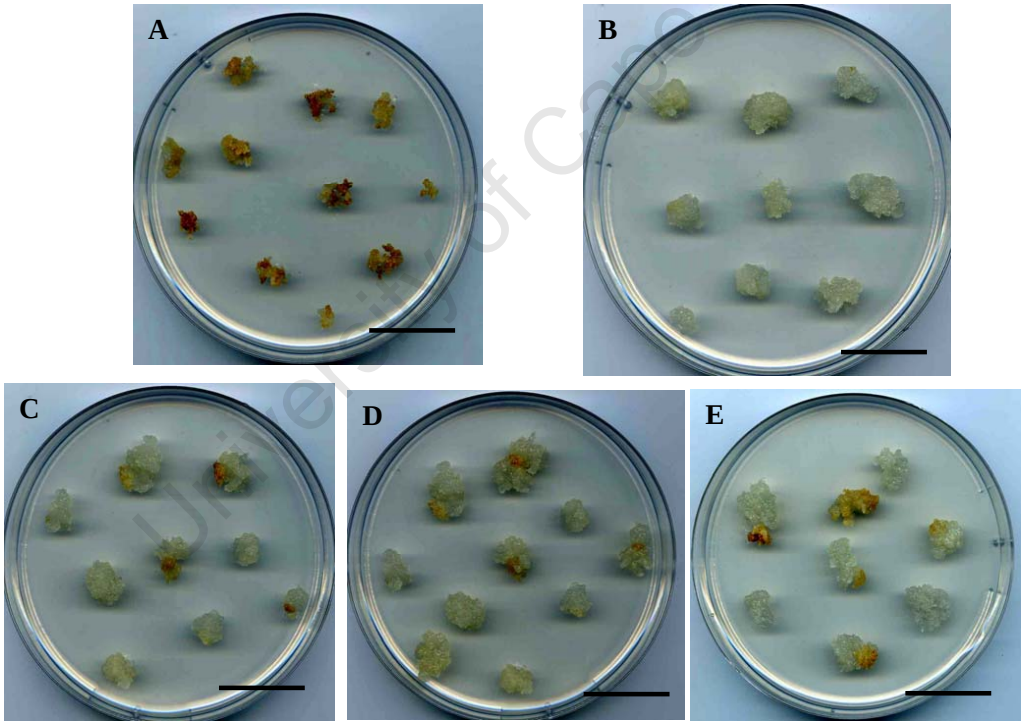

Figure 3.3: Putative BMS transformants 4 weeks post bombardment under bialaphos selection. **A**: Non-transformed BMS cells on selection media containing 3 mg/l bialaphos displaying total necrosis. **B**: Non-transformed BMS cells on media containing no bialaphos. **C**, **D**, and **E**: BMS cells transformed with the *luc* gene under control of the XvPsap1, XvPsap2, and XvPsap3 promoter fragments, respectively on selection media containing 3 mg/l bialaphos (scale bars = 2.5 cm).

3.4.4 PCR screening of putative transgenic BMS cells

Following 5 weeks of selection, bialaphos resistant BMS cell lines were analysed for the presence of *luc* transgenes by PCR screening. The majority of cell lines were positive for the expected 1.2 kb luciferase gene fragment (Fig. 3.4 A). Interestingly, some cells that survived selection lacked the presence of the *luc* gene (Fig. 3.4 A). Consequently, further screening of these lines was conducted using *bar* specific primers. The expected 421 bp *bar* fragment was amplified in all cell lines (Fig. 3.4 B).

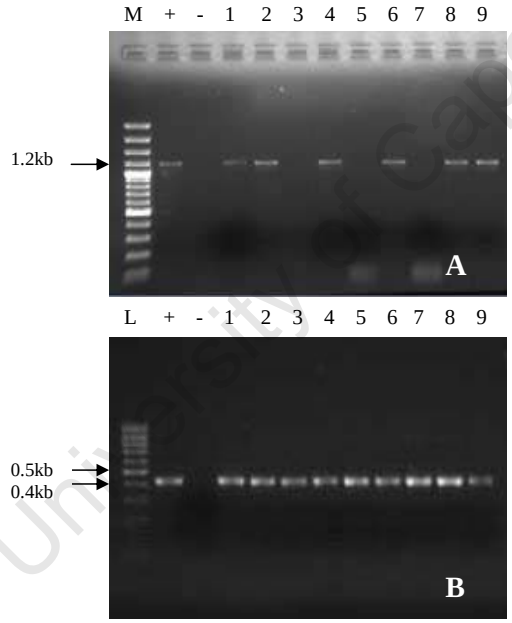

Figure 3.4: PCR screening of transgenic BMS cells for the presence of the *luc* and *bar* transgenes. **A**: The expected 1.2 kb luciferase fragment was amplified. **M**, 100 bp DNA ladder Plus (Fermentas, Canada); +, 1 ng of pA53 containing the *luc* gene; **-**, non-transformed BMS cells. Cells transformed with XvPsap1 (lanes 1-3), XvPsap2 (lanes 4-6) and XvPsap3 (lanes 7-9) constructs. **B**: The expected 421 bp *bar* fragment was amplified; **L**, 100 bp DNA ladder (Fermentas, Canada); +, 1 ng of pAHC25 containing the *bar* gene.

3.4.5 Southern blot analysis of transgenic BMS cells

To assess the integration of the *luc* transgene in the BMS genome, Southern blot analysis was performed. Success in labeling was confirmed by gel electrophoresis of the DIG labeled-probe. As expected, the labeled probe migrated slower than the unlabeled control due to the presence of the DIG label (Fig. 3.5). The actual size of the tPA amplicon (positive control) is 442 bp, however the presence of DIG slowed its migration hence the expected fragment was between 500-550 bp.

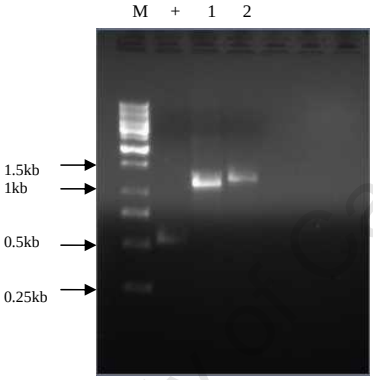

Figure 3.5: Probe synthesis using the PCR DIG probe synthesis kit. **M**, 1 kb DNA ladder (New England Biolabs, USA); +, tissue type plasminogen activator (tPA). Lane 1, unlabeled control probe with expected size of 1.2 kb; lane 2, labeled probe.

Following digestion with *Spe*I and *Bam*HI, the digested genomic DNA was successfully electrophpresed on a 1% agarose gel (Fig. 3.6 A). Capillary transfer onto nylon membrane was successful (Fig. 3.6 B). A single band (1645 bp) representing the *luc* transgene was observed in all samples. The BMS cell lines containing the same copy numbers of the *luc* transgene were predicted by comparing the band intensities given that an equal concentration of DNA was loaded in every well. Accordingly, BMS cell lines marked 4-15 (Fig. 3.6 B) were considered to possess the same copy number and were subsequently used for the luciferase assay. No hybridisation was detected for the non-transformed cell samples (Fig. 3.6 B).

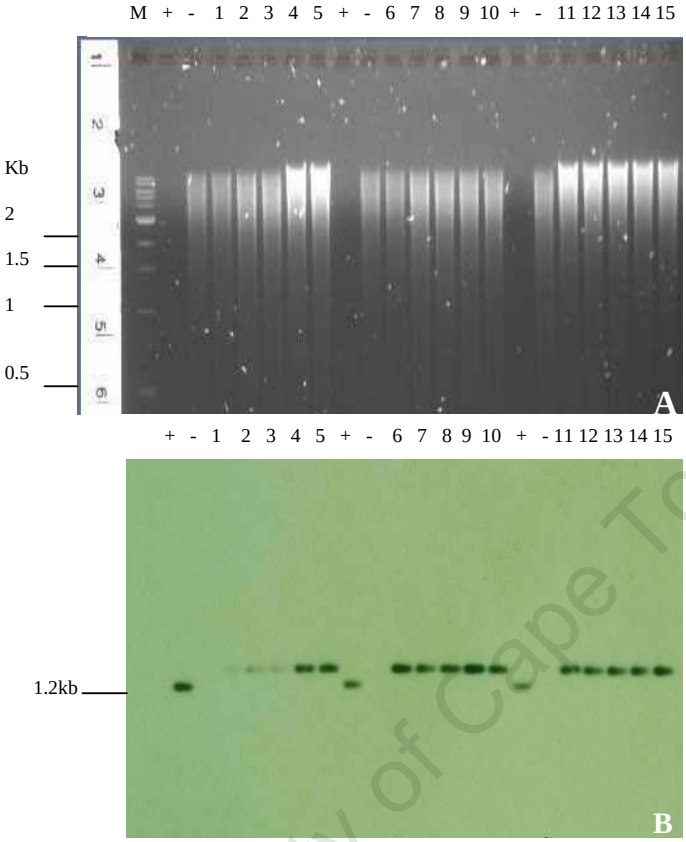

Figure 3.6: Southern blot analysis of transgenic BMS cell lines for the presence of *luc* transgene. **A**: Digested genomic DNA separated on a 1% agarose gel. **B**: Autoradiograph of membrane probed with a DIG-labeled probe specific to a 1.2 kb fragment of the *luc* gene. **M**, 1 kb DNA ladder (New England Biolabs, USA); Cell lines transformed with the XvPsap1 (lanes 1-5), XvPsap2 (lanes 6-10), and XvPsap3 (lanes 11-15) constructs. +, 200 pg of the 1.2 kb PCR *luc* fragment used to synthesise the probe. **-**, digested genomic DNA of non-transformed cell lines.

3.4.6 Luciferase activity in primary BMS cell transformants

Transgenic BMS cells from independent transformation events were examined to determine the levels of luciferase activity following 200 mM NaCl stress. Some transformation events yielded more than one transgenic clone due to the way the cells were split up during subculture. The BMS cells transformed with XvPsap1 displayed the highest activity (3-fold) following 24 h of salt stress (Fig. 3.7A). The XvPsap2 and XvPsap3 fragments displayed lower activities of 1.4- and 1.2-fold,

respectively (Fig. 3.7A). The MTT assay conducted after 24 h of stress indicated a percentage survival of greater than 60% for all the promoter fragments (Fig. 3.7B). After 48 h and 72 h of salt stress, when cell survival was barely above 50%, luciferase activity dropped dramatically to almost undetectable levels in all the transgenic cell lines (Fig. 3.7 A, B). While varying luciferase activity was recorded for the different promoter fragments, these values were not significantly different ($P < 0.05$).

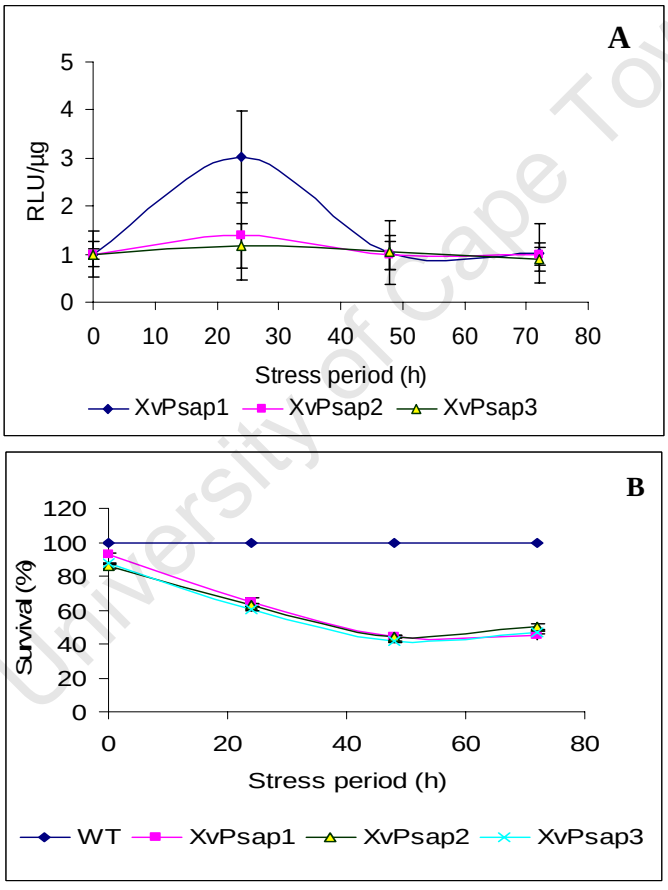

Figure 3.7: Luciferase activity in transformed BMS cell lines treated for 72 h with 200 mM NaCl. **A**: Luciferase activity in transgenic BMS cells. **B**: Percentage survival of transgenic BMS cells. The data are presented as means ± SEM from three independent transgenic BMS cells.

3.4.7 Analysis of *luc* expression in transgenic BMS cells using qRTPCR

The qRTPCR analysis of BMS cells pointed to the *luc* mRNA being upregulated within 24 h of salt stress for both XvPsap1 (5-fold; $P < 0.05$) and XvPsap2 (1.9-fold; $P < 0.05$) whereas with XvPsap3 (1.9-fold; $P < 0.05$) upregulation occurred after 48 h (Fig. 3.8). Longer salt stress periods of up to 72 h did not induce *luc* expression as the transcripts rapidly declined to undetectable levels.

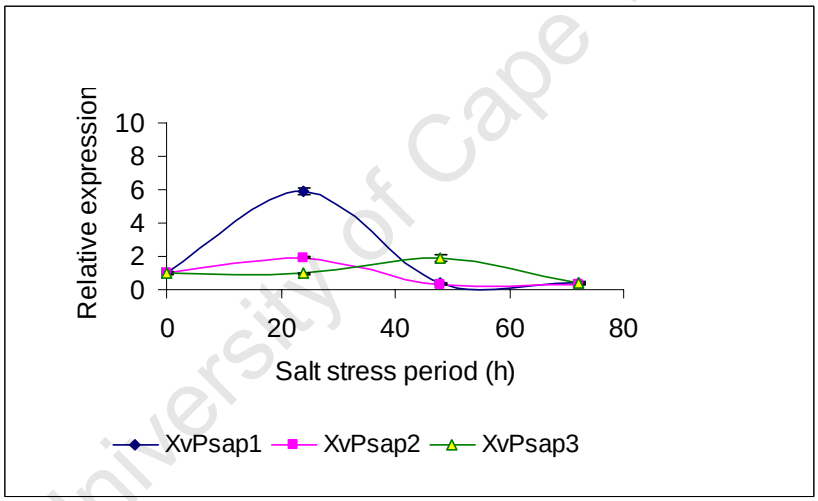

Figure 3.8: Luciferase expression profile curves in transgenic BMS cells following treatment over a 72 h period with 200 mM NaCl. The data are presented as means ± SEM from three independent transgenic BMS cells.

3.5 Discussion

In the present study, the growth pattern of BMS cells under salt stress was determined using the MTT assay (Plumb et al. 1989). The assay has been widely adopted following the findings of Plumb et al. (1989) who reported the establishment of conditions under which MTT reduction could be used quantitatively to determine surviving cell numbers in a chemosensitivity assay that could be applied to both adherent and non-adherent cell lines.

Common data variations usually encountered when experimenting on BMS cells were never observed in this study. A plausible explanation is that since cell samples were collected at the same time point during the day, any variation due to circadian rhythms was minimised. In addition, the concentration of MTT and D-glucose as well as the culture pH were standardised (Plumb et al. 1989). This is necessary since MTT reduction correlates well with D-glucose concentration in the medium at the time of assay. Cell lines which metabolise the sugar extensively tend to exhibit the greatest decrease in the production of MTT formazan (Vistica et al. 1991) thus leading to variations in the data output.

Salt stress was observed to inhibit the growth of BMS cells. This observation could be attributed to the fact that salt stress causes inhibition of plant growth due to a reduction in water availability, sodium ion accumulation, and mineral imbalances leading to cellular and molecular damage (Silva-Ortega et al. 2008). The growth reduction is a specific characteristic of salt sensitive plants. However, this characteristic has also been identified in higher plants such as *Opuntia* subjected to months of salt stress (Murillo-Amador et al. 2001, Silva-Ortega et al. 2008).

The jellyfish green fluorescent protein and the firefly luciferase protein are two commonly used molecular reporters that can be detected non-invasively in living cells. The properties that make GFP or Luc useful for a particular experimental application are quite distinct. Thus a recombinant protein with both fluorescent

and bioluminescent characteristics might take advantage of the strengths of both reporter genes (Day et al. 1998).

In the present study, GFP fluorescence was observed in the salt treated transgenic BMS cells transformed with the three promoter fragments. The differential activity of the individual promoter fragments could not be elucidated from the GFP florescence observed due to lack of equipment for quantifying GFP fluorescence. Nevertheless, the data served a useful function as a preliminary visual indicator of promoter activity. The GFP fluorescence was insignificant following 72 h of salt stress. This decline points to the fact that the XvPsap1 promoter is involved in early responses to abiotic stress (Iyer et al. 2007). Recently, the expression of the *gfp* gene has been reported to peak 24 h post introduction followed by a rapid decline thereafter (Chiera et al. 2008). Such declines in expression, have previously been attributed to pre-integrative DNA events that involve the loss of introduced DNA or cell death (Chiera et al. 2008). However, Chiera et al. (2008) ascribe the declines to post-transcriptional gene silencing. The GFP fluorescence was extended to well over 100 h when GFP was expressed as a translational fusion to the RNA silencing suppressor protein HCPro from tobacco etch potyvirus (Chiera et al. 2008).

Selectable marker genes that confer resistance to antibiotics or herbicides are generally incorporated along with the gene of interest in most plant transformation systems to allow transgenic material to be identified and to give transgenic cells the chance to proliferate without being overgrown by non-transformed cells (Harwood et al. 2002, Jaiwal et al. 2002). The use of the *bar* gene, which confers tolerance to bialaphos effectively inhibited the growth of non-transformed BMS cells. When bialaphos resistant BMS cells were sreened for presence of the *bar* gene, all tested positive. In contrast, when the same cell samples were assessed for the presence of the *luc* transgene, some tested negative. This observation could be linked to the drawbacks associated with biolistic transformation in terms of stability and integration of transgenes (Shrawat 2007) as well as low transformation efficiency

in comparison to other transformation strategies (Sharma et al. 2005). Moreover, because this study employed co-bombardment, it is likely that some BMS cell lines only received the pAHC25 plasmid (that harbours the *bar* gene) and not the pA53 plasmid (that contains the *luc* transgene) resulting in the observed bialaphos tolerance. Similar observations have been reported in whole plant transformation in different studies. For instance, while assessing the application of gene silencing as a tool for development of resistance to maize streak virus, Owor (2008) noted that some of the transgenic *Digitaria sanguinalis* and maize generated that were positive for the *bar* gene, tested negative for the *rep*ΔI^{678} transgene.

Stable introgression of the *luc* transgene was confirmed by Southern blot analysis. Varying transgene copy numbers has been reported to cause instability as well as variation in transgene expression (Finnegan & McElroy 1994). In this study, such variations in luciferase activity and qRTPCR were minimised by using only those cell lines predicted to possess the same gene copy numbers.

Of the three promoter fragments, XvPsap1 (2083 bp) recorded the highest luciferase activity of 3-fold followed by XvPsap2 and XvPsap3 in descending order of activity. The fact that luciferase activity peaked at 24 h and was followed by a rapid decline thereafter, strongly suggests that the XvPsap1 promoter is involved in early response to stress. Significantly, the XvPsap1 sequence was isolated upstream of the *XvSap1* gene, which Garwe et al. (2006) reported to confer tolerance to dehydration, high temperatures and salinity in transgenic *A. thaliana*.

While these results provide a profound understanding of the activity of the promoters, it should be noted that luciferase activity can be affected by several factors. The fact that the luciferase reaction is ATP-dependent suggests that the reaction output is prone to variations depending on the energy status of the cells at the time of the assay. Luciferase can also be degraded by proteases. However, protein inhibitor was used in the present study to prevent such degradations. Furthermore, when living cell cultures stably expressing the GFP::Luc fusion were treated with

the protein translation-inhibitor cycloheximide, the half-life for luciferase protein activity was determined to be approximately 2 h at 37°C (Day et al. 1998). This suggests that the protein is somewhat stable.

In light of these possible sources of variation, the activity of the XvPsap promoters was further analysed by performing qRTPCR. The XvPsap1 promoter recorded the highest levels of *luc* transcripts following 24 h of salt stress. Previously, Iyer et al. (2007) reported a similar trend with the expression of the *XvSap1* gene in dehydrated *X. viscosa*, which is believed to be naturally driven by the XvPsap1 promoter. They noted an up-regulation of the *XvSap1* mRNA at 60% RWC. Thereafter, expression decreased but again increased at 15% RWC. This observation led Iyer et al. (2007) to conclude that *XvSap1* could be involved in the initial and late stages of the protective response to dehydration. The discrepancy observed between the transcriptional (5-fold) and translational (3-fold) levels could be attributed to factors including but not limited to *luc* mRNA degradation, translational control as well as post-translational modification (Gallie 1996, Yanagisawa 1998). Together, these results clearly demonstrate the differential activity of XvPsap promoters in BMS cells under salt stress.

The end point of a chemosensitivity assay is usually an estimate, either direct or indirect, of surviving cell numbers. It is therefore uncertain that the expression trends reported in this study would apply to the whole plant system, particularly to dicots. Furthermore, use of particle bombardment for transformation presents notable drawbacks. Consequently, transformation of *N. tabacum* using the same gene constructs by *Agrobacterium*-mediated transformaton is reported in chapter 4.

Chapter 4

Functional analysis of XvPsap promoter activity in transgenic *Nicotiana tabacum*

4.1 Summary

The chimeric constructs harbouring XvPsap1 and its truncated fragments regulating the expression of *luc* were used to transform *N. tabacum* by *Agrobacterium*-mediated transformation. The functional properties of each promoter fragment was examined by fluorescence quantitative analyses of transgenic tobacco leaves following dehydration stress for eight days. Of the three promoter fragments, XvPsap1 was the most active with optimal activity attained after three days of dehydration stress. The qRTPCR analysis confirmed the upregulation of the *luc* gene (7-fold; $P< 0.05$) under control of XvPsap1 within three days of stress whereas XvPsap2 and XvPsap3 displayed minimal activities of 2.2- and 1.6-fold, respectively. These results are significant in that the XvPsap1 promoter displays activity in a dicot system although it was isolated from a monocotyledonous system. This suggests that the promoter has added potential for use in the generation of transgenic dicotyledonous crops tolerant to drought stress.

4.2 Introduction

Over the past 30 years, *N. tabacum* has been used as a key plant model for the development of transformation technology and molecular analyses. This probably reflects the fact that tobacco was the first plant species to be regenerated *in vitro* (Skoog & Miller 1957) and was used to develop standardised tissue culture conditions (Murashige & Skoog 1962). Tobacco plants are ideal for biotechnology procedures because it is leafy, readily accepts the procedures, grows quickly, is relatively easy to harvest, and yields millions of seeds per plant. Consequently, tobacco has become a preferred system for the expression of genes following *Agrobacterium*-mediated transformation (Florack et al. 1994, Kasuga et al. 2004).

The transformation of *N. tabacum* using *luc* constructs described in chapter 2, through *Agrobacterium*-mediated transformation is reported. The expression and relative activity of luciferase is also described. The main objectives were: (i) to determine whether the XvPsap promoter fragments are functional in a dicot system; and (ii) to determine the most active promoter fragment in a model dicot system.

4.3 Materials and Methods

4.3.1 Source of explants and surface sterilisation

All *N. tabacum* seeds were surface sterilised for 2 min in 70% ethanol followed by soaking for 15 min in 3.5% commercial bleach (active ingredient 2.5% w/v sodium hypochlorite) supplemented with a single drop of wetting agent (Tween 20). The bleach treatment was repeated once before rinsing the seeds six times with sterile distilled water.

4.3.2 Seed germination and growth conditions

To promote even germination on plates, the sterile seeds were stratified in 0.1% agar at 4°C prior to germination. The seeds were germinated and propagated on germination medium comprising 4 g/l MS medium supplemented with 10 g/l sucrose, 2 mg/l glycine, 0.5 g/l 2- (4-morpholino)-ethane sulfonic acid and 0.8% agar. The pH was adjusted to 5.7.

Unless stated otherwise, all growth regulators, herbicides and antibiotics were filter sterilised and freshly added to the media following autoclaving. All plants were grown under the following conditions except where stated otherwise: 16 h day, 8 h night, 24°C and 65% humidity. Once the tobacco seeds germinated the seedlings were transferred onto fresh media in culture bottles.

4.3.3 Transformation of *N. tabacum*

4.3.3.1 Gene constructs

The chimeric pTF101.1 constructs described in chapter 2, carrying the *luc* gene in the sense orientation, were transformed into *A. tumefaciens* strain EHA101 by the freeze-thaw method (An et al. 1988). The transformation of tobacco leaf discs was

according to the procedures outlined by Horsch et al. (1985). The recombinant bacteria cultures were selected on YEP media comprising 5 g/l yeast extract, 10 g/l peptone, 5 g/l sodium chloride, 100 mg/l spectinomycin, 50 mg/l kanamycin and 30 mg/l chloramphenicol. The pH was adjusted to 6.8.

4.3.3.2 Pre-induction of *A. tumefaciens*

Freshly cultured *A. tumefaciens* was resuspended in filter sterilised infection media. The infection media comprised MS medium supplemented with 30 g/l sucrose, 2 mg/l glycine and 100 μM/l acetosyringone. The pH was adjusted to 5.2. Cell cultures were incubated in the dark at 30°C in a shaker until an optical density (650 nm) of 0.6 was attained.

4.3.3.3 Infection of explants

Sterile tobacco leaf discs were pricked prior to slicing into uniform segments. The leaves were immediately submerged in infection media to minimise wilting. The leaf discs were infected for 30 min in the dark and thereafter blot dried on sterilised filter paper. Negative controls infected with *A. tumefaciens* containing pTF101.1 vector were also included.

4.3.3.4 Co-cultivation of infected explants

Each infected explant was transferred to co-cultivation medium and incubated for three days in the dark at 19°C with the adaxial part of the leaf in contact with the medium. The co-cultivation media comprised MS basal salts supplemented with B5 vitamins, 30 g/l sucrose, 0.1 mg/l α-naphthaleneacetic acid, 1 mg/l 6-benzylamino purine and 100 μM/l acetosyringone. For co-cultivation, shooting and rooting media the pH was adjusted to 5.9.

4.3.3.5 Selection of putative tobacco transformants

Following co-cultivation, leaf discs were selected on shooting medium comprising MS basal salts supplemented with B5 vitamins, 30 g/l sucrose, 0.1 mg/l α-naphthaleneac-
etic acid, 1 mg/l 6-benzylamino purine, 250 mg/l carbenicillin and 2 mg/l bialaphos. Putative transformants were subcultured fortnightly onto fresh medium until sizable shoots were formed. Bialaphos resistant shoots were selected and transferred to root induction medium. The rooting media comprised half strength hormone-free MS basal salts supplemented with 10 mg/l sucrose, 250 mg/l carbenicillin and 1 mg/l bialaphos.

4.3.4 Acclimatisation and growth of putative tobacco transformants

Putative transformants were transferred to pots containing sterile peat moss. The plants were covered using transparent plastic bags to minimise dehydration. The plants were acclimatised for eight days prior to transplanting to soil. The putative transformants were grown in a greenhouse at 26°C under natural lighting supplemented with fluorescent lamps to maintain the 16 h photoperiod. The mature transgenic plants were self-pollinated and seeds were harvested. Transformation efficiency was calculated as follows:

$$\text{Transformation Efficiency} = \left\{ \frac{\text{No. of Positive Transformants}}{\text{No. of Explants Transformed}} \right\} \text{x}\, 100$$

4.3.5 Germination of putative transgenic tobacco seeds

Seeds of putative tobacco transformants were surface sterilised (see section 4.3.1) and plated on germination medium. The germination media comprised MS media supplemented with 2 mg/l glycine, 0.5 g/l sucrose, 8 g/l agar and 2 mg/l bialaphos.

The pH was adjusted to 5.7. Only bialaphos-resistant seedlings were transplanted to pots for further screening.

4.3.6 Basta screening of putative tobacco transformants

Basta screening was conducted on putative transformants. Basta (0.75%) was supplemented with a single drop of Tween 20 and painted directly onto leaves. Herbicide resistance was scored 5-7 days following herbicide application.

4.3.7 Detection and determination of transgene integration

The presence of *luc* and *bar* transgenes was detected by PCR amplification (see section 3.3.4). Stable introgression of the *luc* transgene was determined by Southern blot analysis. The restriction endonucleases, *Spe*I and *Bam*HI were used to digest the genomic DNA overnight at 37°C. Hybridisation and detection of probe was carried out using a non-radioactive, DIG Luminescent Detection Kit for nucleic acids (see section 3.3.5). To detect *Agrobacterium* contamination in plant tissues, the primer pair VCF and VCR (Sawada et al. 1995) with expected amplimer size of 730 bp was used to detect the bacterial *vir*C gene. The amplification conditions was as follows: 94°C for 150 s; 35 cycles of 94°C for 60 s; 55°C for 60 s, and 72°C for 120 s; and a final extension step of 72°C for 7 min.

4.3.8 Dehydration treatment of transgenic tobacco

Dehydration stress was imposed by withholding water for eight days. Thereafter stressed tobacco leaves were harvested at 0, 3, 6 and 8 days. Leaf samples from at least three independent T_2 homozygous tobacco transformants were immediately frozen in liquid nitrogen upon harvesting and stored at -70°C.

4.3.9 Determination of relative water content

The relative water content (RWC) was determined for leaf samples at each sampling time point. The initial weight (W_{ini}) of each sample was determined prior to immersion in sterile distilled water. Weight at full turgor (W_{ft}) was determined following 24 h incubation in water. Leaf samples were dried for 2 days at 70°C prior to the dry weight (W_d) being determined. The formula described by Jin et al. (2000) was used to calculate the RWC:

$$\text{RWC} = \frac{(W_{ini} - W_d)}{(W_{ft} - W_d)} \text{ x } 100$$

4.3.10 Total RNA extraction, purification and cDNA synthesis

Total RNA was isolated from leaf samples using the TRIzol method (see section 3.3.8). Thereafter, total RNA was purified using the EZ-10 Spin Column Total RNA Minipreps Super Kit followed by one step cDNA synthesis using random hexamers (see section 3.3.9).

4.3.11 Analysis of luciferase activity and qRTPCR

Protein was extracted from three dehydrated independent T_2 homozygous tobacco leaf samples (see section 3.3.7). Thereafter, luciferase activity was measured using a Modulus Microplate Multimode Reader (see section 3.3.7). The protein concentration of the extract was determined using the Braford reaction and luciferase activity was expressed as RLU/μg protein. The relative expression of the *luc* transgene was determined using qRTPCR (see section 3.3.10). Promoter activity data was analyzed for significant difference (see section 3.3.11).

4.4 Results

4.4.1 Tissue culture, transformation and growth of putative transformants

Tobacco seeds used in this study were determined to be viable as germination was observed within one week after plating onto germination media (Fig. 4.1 A). Tissue culture of tobacco was successful and no growth hormone was required for propagation (Fig. 4.1 B). Following incubation for three days on co-cultivation media, the infected leaf discs started to brown (Fig. 4.1 C). When non-transformed leaf discs were transferred to shooting media containing 2 mg/l of bialaphos, total necrosis was observed (Fig. 4.1 D). In contrast, shoots emerged on the transformed explants with only partial necrosis observed in isolated areas of the explants (Fig. 4.1 E). The shoots only developed roots after being transferred to the rooting media with reduced bialaphos concentration (1 mg/l; Fig. 4.1 F). Successful acclimatisation of the transformants was observed with all transformants surviving the hardening process (Fig. 4.1 G). The putative transformants did not display any abnormal phenotypes and grew normally to maturity (Fig. 4.1 H). The pollination was controlled (Fig. 4.1 I) and the transformants were self-pollinated thereafter.

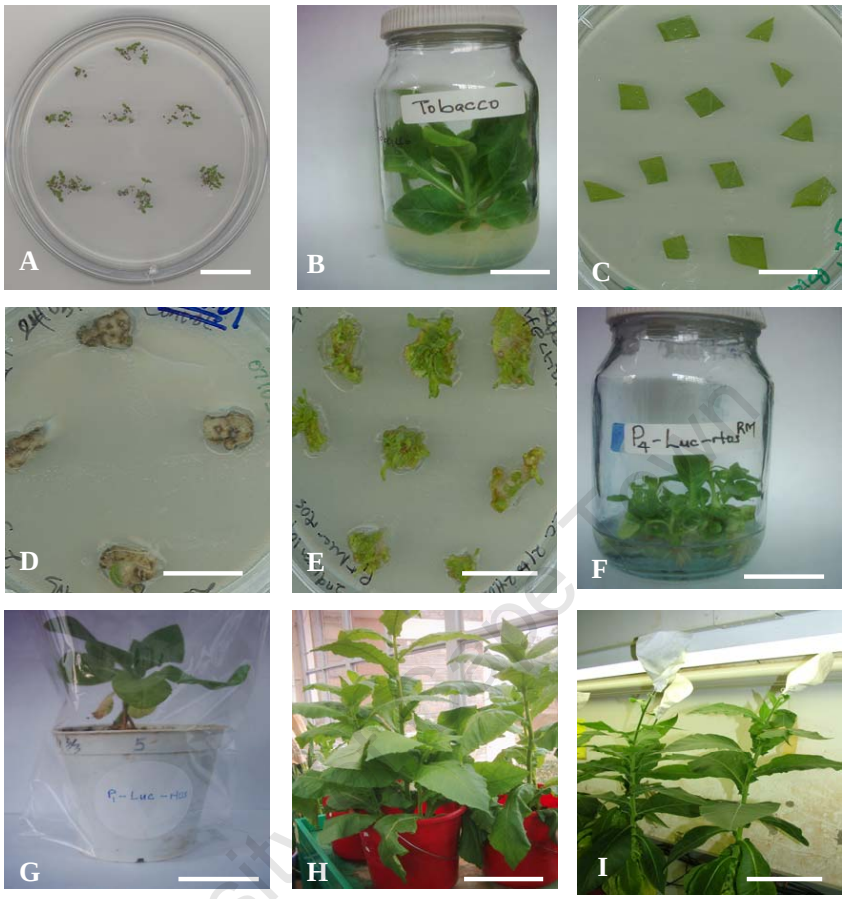

Figure 4.1: Transformation and tissue culture of *N. tabacum*. **A**: Non-transformed sterile tobacco seeds after 1 week on germination media. **B**: Non-transformed tobacco that served as the source of explant cultured on hormone-free propagation media. **C**: Transformed tobacco leaf discs on co-cultivation media. **D**: Non-transformed tobacco leaf discs 3 weeks after infection showing total necrosis on shooting media. **E**: Shoots emerging from transformed leaf discs with partial necrosis observed on non-transformed areas after 3 weeks on shoot selection media. **F**: Transformants forming roots on rooting media 5 weeks after infection. **G**: Transformants covered with polythene bags to minimise water loss during hardening. **H**: Putative transformants growing to flowering in glass house. **I**: Flowers of putative transformants covered with pollination bags (scale bars: **A-E** = 2 cm; **F-G** = 3 cm; **H-I** = 20 cm).

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4.4.2 Germination of putative transgenic tobacco seeds

Following sterilisation, the seeds of the putative transformants were successfully plated on germination media supplemented with 2 mg/l bialaphos (Fig. 4.2 A).

Interestingly all seeds, including the non-transformed seeds, germinated (Fig. 4.2 B). However, when the seedlings were further incubated for 3 weeks on germination media, the non-transformed seedlings displayed total necrosis (Fig. 4.2 C). The transformants of XvPsap1, XvPsap2 and XvPsap2 displayed partial survival on bialaphos containing media (Fig. 4.2 D, E, F), respectively. Only transformants that germinated and developed at least four rosette leaves were regarded as resistant.

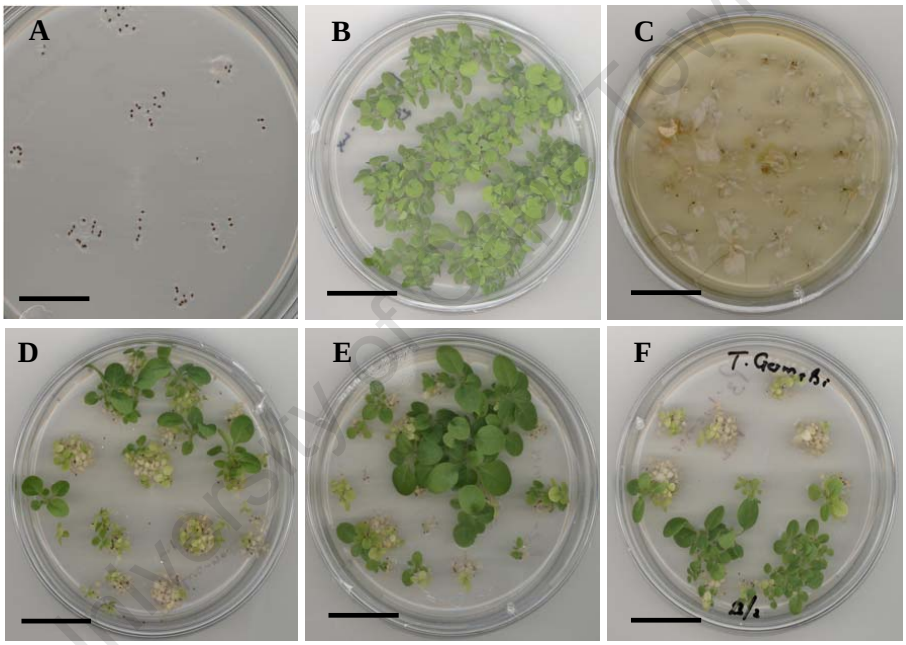

Figure 4.2: Germination of transformed *N. tabacum* seeds. **A**: Transformed tobacco seeds 1 day after plating. **B**: Non-transformed seedlings 2 weeks after plating. **C**: Non-transformed seedlings showing total necrosis 3 weeks after plating. **D**, **E**: and **F**: Seedlings transformed with XvPsap1, XvPsap2 and XvPsap3 promoter fragments, respectively showing partial necrosis 3 weeks after plating (scale bars = 2 cm).

4.4.3 Basta screening of putative tobacco transformants and PCR detection

Non-transformed tobacco seedlings were observed to be susceptible to basta screening whereas transformed plants displayed tolerance (Fig. 4.3). The presence of the *luc* transgene was observed following amplification with the expected 1.2 kb fragment of the gene being detected (Fig. 4.4 A). All transformants that tested positive for the *luc* transgene also showed the presence of the *bar* transgene (Fig. 4.4 B). Transformation efficiency was greater than 55% for all constructs and there was no significant difference among individual constructs (Table 4.1).

Table 4.1: Transformation efficiency of *N. tabacum*

Construct	No. of transformation events	No. of infected explants	No. of positive transformants	Transformation efficiency ± SE*
XvPsap1::luc	5	560	370	66±6.3[a]
XvPsap2::luc	6	700	406	58±9.3[a]
XvPsap3::luc	5	520	312	60±5.0[a]

*SE: Standard deviation of the mean of transformation events

[a]: No significant difference among the gene constructs, $P < 0.05$

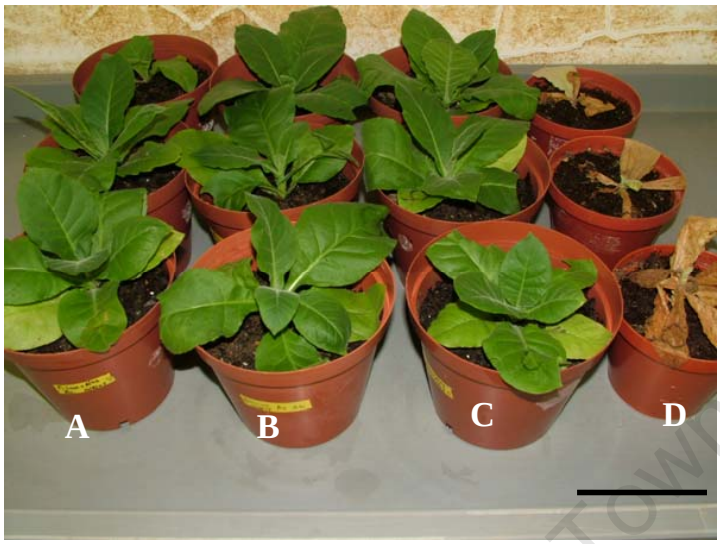

Figure 4.3: Basta selection of putative *N. tabacum* tansformants. Columns **A**, **B** and **C**: Plants transformed with XvPsap1, XvPsap2 and XvPsap3, respectively. **D**: Non-transformed plants (scale bar = 15 cm).

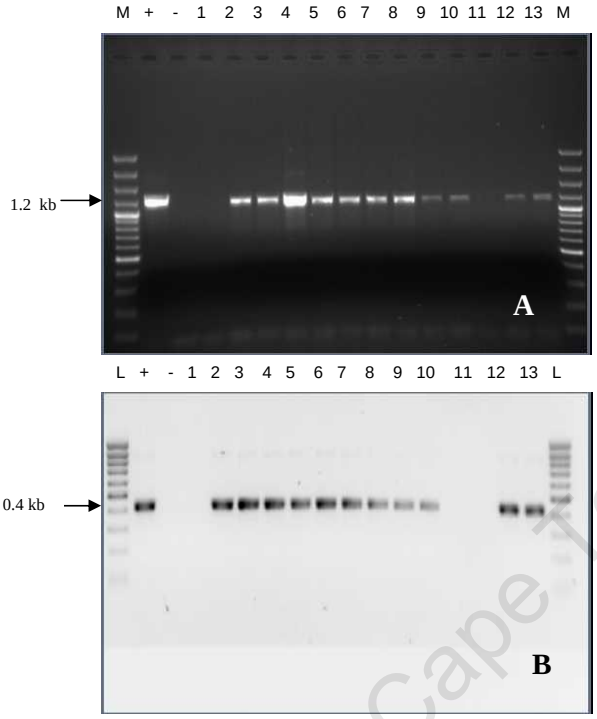

Figure 4.4: Screening of putative transgenic *N. tabacum* by PCR for the presence of the *luc* (**A**) and *bar* (**B**) transgenes. **A**: The expected 1.2 kb fragment of the *luc* gene was amplified. **M**; 100 bp DNA ladder plus (Fermentas, Canada). +: 1 ng of pTF101.1 containing the *luc* gene. -: non-transformed *N. tabacum*. Plant transformed with XvPsap1 (lanes 1-5), Xvsap2 (lanes 6-9), and XvPsap3 (lanes 10-13) constructs. **B**: The expected 421 bp fragment of the *bar* gene was amplified. **L**: 100 bp DNA ladder (Fermentas, Canada).

4.4.4 Southern blot analysis

Southern blot analysis of transgenic *N. tabacum* genomic DNA probed with a 1.2 kb fragment of the *luc* gene was performed to confirm the presence of the *luc* transgene in the genome of transgenic plants and also to estimate gene copy number. The *Spe*I and *Bam*HI restriction endonucleases were successfully used in the digestion of tobacco genomic DNA. The digested genomic DNA was successfully electrophoresed on a 1% agarose gel (Fig. 4.5 A). Capillary transfer onto nylon membrane was also successful (Fig. 4.5 B). The expected single band (1645 bp) representing the *luc* transgene was observed in all the samples. The non-transformed

plants showed no hybridization product. Plants possessing transgenes at similar copy numbers were predicted by comparing band intensities and patterns.

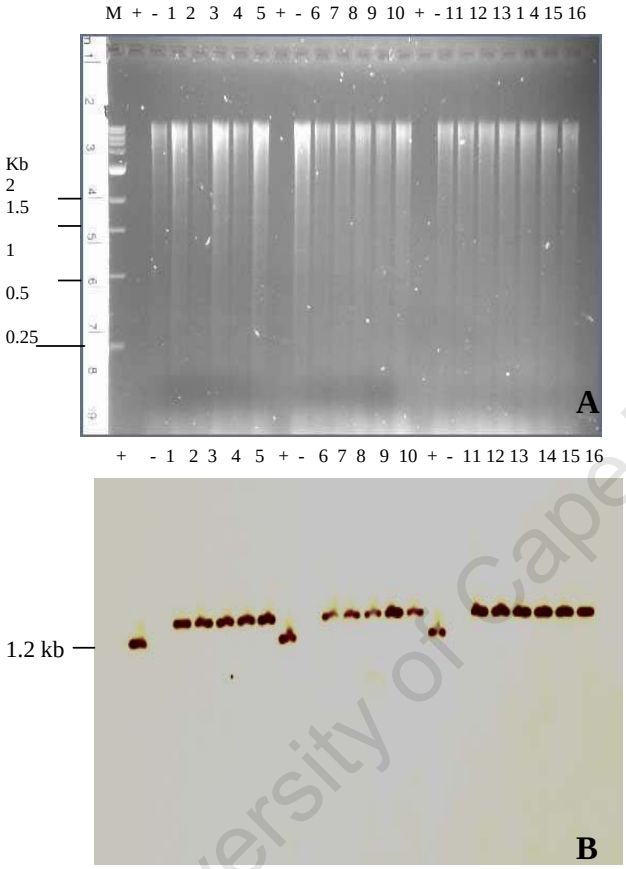

Figure 4.5: Southern blot analysis of transgenic *N. tabacum* for the presence of the *luc* transgene. **A**: Digested genomic DNA separated on a 1% agarose gel. **B**: Autoradiograph of membrane probed with a DIG-labeled probe specific to a 1.2 kb fragment of the *luc* gene. **M**: 1 kb DNA ladder (New England Biolabs, USA). Plants transformed with XvPsap1 (lanes 1-5), XvPsap2 (lanes 6-10), and XvPsap3 (lanes 11-16) constructs. **+**: 200 pg of the 1.2 kb PCR *luc* fragment used to synthesise the probe. **-**: genomic DNA of non-transformed tobacco.

4.4.5 Analyses of luciferase activity in transgenic plants

Following protein extraction from dehydrated tobacco leaf samples, luciferase activity was determined. The XvPsap1 promoter recorded the highest luciferase activity (3.97-fold; Fig. 4.6 A). The plants transformed with XvPsap2 and XvP-

sap3 demonstrated lower luciferase activities of 1.4- and 1.3-fold, respectively (Fig. 4.6 A). For all the plants, luciferase activity was observed to be optimal shortly after initiation of the dehydration stress with RWC above 70% (Fig. 4.6 B)

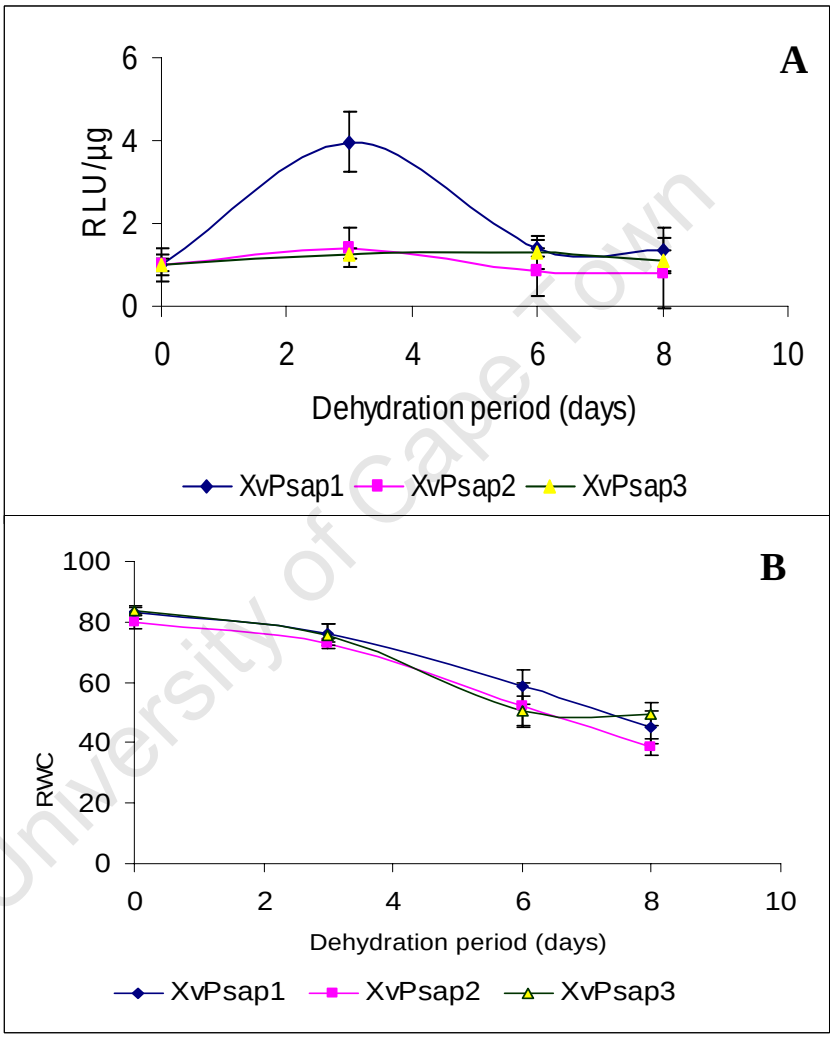

Figure 4.6: Luciferase activity (**A**) and relative water content (**B**) in transgenic *N. tabacum* under dehydartion stress. The data are presented as means ± SEM from three independent homozygous tobacco transformants.

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4.4.6 Analyses of transgenic plants by qRTPCR

The ability of the promoter fragments to express the *luc* gene under dehydration stress was determined by qRTPCR. Optimal expression levels were observed three days after initiating the dehydration stress. The increase in XvPsap1 activity was significant (7-fold; $P < 0.05$) in transgenic tobacco whereas XvPsap2 and XvPsap3 displayed lower increases of 2.2- and 1.6-fold, respectively.

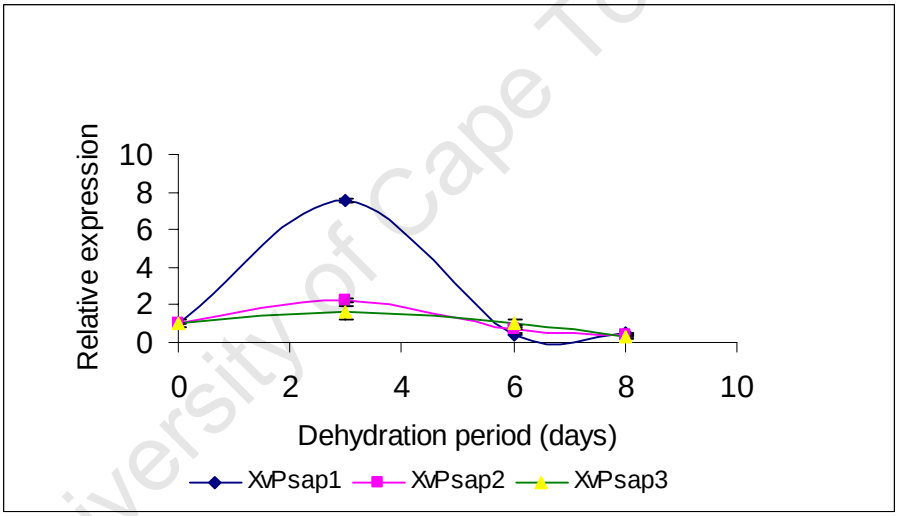

Figure 4.7: Expression profile curves of *luc* transcripts in transgenic *N. tabacum* following dehydration treatment. The data are presented as means ± SEM from three independent homozygous tobacco transformants.

4.5 Discussion

Transcription factors or trans-acting factors are capable of specifically binding with *cis*-acting elements in the promoter domains located upstream of genes. These factors therefore control the expression of downstream genes and regulate various physiological and biochemical reactions (Kasuga et al. 2004). In this study, the activity of the three XvPsap promoter fragments was demonstrated in *N. tabacum*. Of the promoter fragments, XvPsap1 displayed the highest activity. Optimal activity was recorded shortly after initiation of dehydration when RWC was above 70%. This suggests that XvPsap1 may be involved in early response to abiotic stress. This observation is consistent with the XvPsap1 activity in BMS cells reported in chapter 3. Given that the promoter originated from a monocot system, the observed activity in tobacco clearly points to the fact that the XvPsap1 promoter has overcome possible cross-species hindrances. This is not unique. In previous studies, trans-species application of promoters has been reported. For example, Shiqing et al. (2005) reported the successful application of the stress-inducible *rd29A* promoter from *Arabidopsis* in generating transgenic wheat tolerant to drought and salt stress.

Transgenic plants generated in this study within the same transformation event, were observed to exhibit varying promoter activity. This phenomenon is correlated to a variety of factors such as uncertain insertion site of the transgene in the genome, instability of gene expression caused by multiple copy numbers (Finnegan & McElroy 1994), as well as DNA methylation which ultimately causes transgenic silence (Srivastava et al. 1999).

Bialaphos was used in this study to select the putative transformants. Interestingly, the seeds of both the transformed and non-transformed controls also germinated on media containing bialaphos. However, longer incubation periods on the same media inhibited the growth of non-transformed plants. This observation is attributable to the fact that bialaphos is a unique tripeptide composed of alanylalanine and phos-

phinothricin joined by a C-P-C bond (Kunitaka 2003), wherein phosphinothricin inhibits glutamine synthetase, accumulates ammonia, and inhibits photosynthesis in plants (White et al. 1990).

Since tobacco is susceptible to *Agrobacterium* infection and has established regeneration protocol (Florack et al. 1994, Kasuga et al. 2004), higher transformation efficiencies of above 60% were achieved in this study. All transgenic plants that tested positive for the presence of the *luc* transgene also showed the presence of the *bar* transgene. This is a significant difference to the findings observed in chapter 3 where BMS cells were transformed via particle bombardment. The fact that the *bar* and *luc* transgenes were cloned within the T-DNA region of a single vector explains the difference in the results obtained. Furthermore, *Agrobacterium*-mediated transformation displays desirable precision in terms of insertional loci (Deroles & Gardner 1988, Cluster et al. 1996, Frame et al. 2002, Vain et al. 2003, Afolabi et al. 2004, Frame et al. 2006, Crowell et al. 2008). For instance, while elucidating the T-DNA integration category and mechanism in the rice genome, Jiang et al. (2005) found that the integrated ends of the T-DNA right border occurred mainly on five nucleotides (TGACA) in inverse repeat sequence of 25 bp, especially on the third base A.

Overexpression of genes has been reported to cause abnormal phenotypes such as dwarfism (Su et al. 1998). For instance, transgenic tomato overexpressing CBF1 demonstrated the dwarf phenotype under unstressed normal growth conditions (Hsieh et al. 2002a,b). However, the use of the stress-inducible *rd29A* promoter instead of the constitutive 35S CaMV promoter for the overexpression of DREB1A in transgenic *Arabidopsis* and tobacco, has been reported to minimise the negative effects on plant growth (Kasuga et al. 1999, 2004). The transgenic tobacco plants generated in this study did not display any significant abnormal agronomic traits compared to the wild type. This may be attributed to the fact that XvPsap1 is stress-inducible.

Overall, this study has demonstrated the activity of XvPsap1 promoter in a model system, namely tobacco. Since this study is part of a larger project that seeks to transform maize for drought tolerance, the activity of the XvPsap1 promoter in maize remains uncertain. As a result, the *Agrobacterium tumefaciens*-mediated transformation of maize is reported in chapter 5.

Chapter 5

Functional analysis of XvPsap1 promoter activity in transgenic *Zea mays*

5.1 Summary

Analysis of the promoter fragments in transgenic BMS cells and *N. tabacum* revealed XvPsap1 as the most active of the three promoter fragments. Consequently, only the XvPsap1 construct was selected for further analyses in maize. Transformation of maize was achieved by *Agrobacterium*-mediated transformation. Southern blot and qRTPCR analyses were conducted on transgenic maize leaves following dehydration for eight days. Luciferase activity peaked (2.2-fold increase) on the third day of dehydration stress and was followed by a significant decline thereafter. The expression profile of *luc* transcripts displayed an optimal expression of 4-fold after three days of stress treatment. The transgenic maize were observed to display normal growth with insignificant phenotypic variations. These results demonstrate that the XvPsap1 promoter is both active and stress-inducible. The XvPsap1 promoter could therefore find application in the generation of transgenic drought tolerant monocot plants.

5.2 Introduction

Maize is mainly used for two purposes in industrialised countries: (i) to feed animals directly in the form of grain and forage or sold to the feed industry; and (ii) as raw material for extractive industries, indicating that maize has little significance as human food (Morris 1998). However, in developing countries especially in sub-Saharan Africa, maize serves as a staple food. Cereals production has been reported to decline in sub-Saharan Africa due to abiotic stresses (Kelemu et al. 2003). In the past, attempts to improve crops for drought tolerance employed conventional breeding (Lamkey 2002). However, with the advances in genetic engineering, accelerated by the limitations of conventional breeding, plant transformation has now gained wide application.

In both transgenic BMS cells (chapter 3) and tobacco (chapter 4) the XvPsap1 promoter was observed to be the most active. Given that this study is part of a larger project that aims to genetically engineer maize for drought tolerance, the focus of this part of the study was to transform *Z. mays* using the XvPsap1 promoter fragment expressing the *luc* transgene by *A. tumefaciens*-mediated transformation. Luciferase expression and relative activity is described. The main objective was to determine the XvPsap1 activity in whole maize.

5.3 Materials and Methods

5.3.1 Source of explants and growth conditions

Maize plants of inbred line (A188) grown either in the field or green house were used as the source of immature embryos. Planting was routinely conducted every three weeks to ensure constant supply of explants. Prompt weeding, top dressing, mulching and regular watering twice a week were performed to promote healthy growth and development of plants. Pollination was controlled (Appendix A.5) and the self-pollinated ears were harvested for transformation experiments.

5.3.2 Surface sterilisation and dissection of immature embryos

Maize ears 13 days post-pollination, with embryo sizes ranging between 1-3 mm in length, were harvested. The ears were either used immediately or stored for a maximum of 3 days at 4°C while still in the husk. Dehusked ears were thereafter surface sterilised for 3 min in 70% ethanol followed by soaking for 18 min in 3.5% commercial bleach (active ingredient 2.5% w/v sodium hypochlorite) supplemented with a singe drop of wetting agent (Tween 20). Ears were rinsed three times with sterile distilled water prior to embryo dissection (Appendix A.6).

5.3.3 Gene construct

The recombinant pTF101.1 vector containing the XvPsap1 promoter expressing the *luc* gene was transformed into *A. tumefaciens* strain EHA 101 by the freeze-thaw method (An et al. 1988). The recombinant bacterial culture was selected on YEP and sodium chloride medium supplemented with the appropriate antibiotics (see section 4.3.3.1). The transformation of immature maize embryos was according to the procedures outlined by Frame et al. (2002) with modifications.

5.3.3.1 Pre-induction of *A. tumefaciens*

The *A. tumefaciens* cultures were resuspended in filter sterilised infection media. The cell cultures were incubated in the dark at 30°C in a shaker until the appropriate bacterial optical density was attained (see section 4.3.3.2). The infection media comprised basal MS salts and modified Linsmaier and Skoog (LS) vitamins (Linsmaier & Skoog 1965) supplemented with 68.4 g/l sucrose, 36 g/l glucose, 2 mg/l 2,4-dichlorophenoxyacetic acid, 1 mg/l casein hydrolysate and filter sterilised 100 μM/l acetosyringone. The pH was adjusted to 5.2. The modified LS vitamin comprised 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 1 mg/l thiamine and 100 mg/l myoinositol.

5.3.3.2 Infection and co-cultivation of immature maize embryos

Immature embryos were immersed for 5 min in pre-induced *A. tumefaciens* in the dark followed by blot drying on sterilised filter paper. Thereafter, the infected embryos were cultured for three days on co-cultivation media in the dark at 19°C, with the embryo axis in contact with the media. The co-cultivation media comprised basal MS salts and modified LS vitamins supplemented with 0.7 g/l proline, 30 g/l sucrose, 10 g/l glucose, 1.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 g/l MES, 8 g/l agar and 100 μM/l acetosyringone. The pH was adjusted to 5.8.

5.3.4 Resting of the infected embryos

Following co-cultivation, the infected embryos were initially washed 3-4 times with sterile distilled water. This was followed by 2 times washing with liquid MS media containing 250 mg/l cefotaxime to eliminate residual bacteria prior to blot drying. The embryos were subcultured on resting media and incubated for 10 days in the dark at 26°C. The resting media comprised basal MS salts and modified LS vitamins supplemented with 0.7 g/l proline, 30 g/l sucrose, 1.5 mg/l 2,4-

dichlorophenoxyacetic acid, 0.5 g/l MES, 1.6 mg/l silver nitrate, 8 g/l agar, 250 mg/l cefotaxime and 250 mg/l carbenicillin.

5.3.5 Selection of the putatively transformed calli

Calli were selected for 2 weeks on selection I media in the dark at 26°C. The selection media comprised basal MS salts and modified LS vitamins supplemented with 0.7 g/l proline, 30 g/l sucrose, 1.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 g/l MES, 8 g/l agar, 1.5 mg/l bialaphos, 250 mg/l cefotaxime and 250 mg/l carbenicillin. Thereafter, the bialaphos resistant calli were transferred to selection II media for a period of 6 weeks with fortnightly subculturing to fresh media. Selection II media was identical to selection I media except that bialaphos concentration was doubled to 3 mg/l. The putatively transformed somatic embryos were matured by subculturing on somatic maturation media for 2 weeks in the dark. Somatic maturation media was identical to selection II media except that the sucrose concentration was increased to 60 mg/l and 1 mg/l α-napthaleneacetic acid and 2 mg/l glycine added. The bialaphos resistant calli were transferred to MS hormone-free regeneration media to develop shoots and roots at 26°C under the 16 h light and 8 h dark photo-regime. The MS hormone-free media comprised basal MS salts and modified LS media supplemented with 0.7 g/l proline, 30 g/l sucrose, 2 mg/l glycine, 250 mg/l cefotaxime, 250 mg/l carbenicillin and 8 g/l agar.

5.3.6 Acclimatisation and growth of putative maize transformants

Putative maize transformants were hardened for seven days (see section 4.3.4 for conditions). The transformation efficiency was calculated (see section 4.3.4). The putative transformants were self-pollinated and the seeds harvested.

5.3.7 Basta screening and PCR detection of putative maize transformants

The transformed seeds were germinated in hydrated vermiculite prior to basta treatment (see section 4.3.6). The presence of *luc* and *bar* transgenes was detected by PCR amplification using gene specific primers (see section 3.3.4).

5.3.8 Southern blot analysis

Transgenic maize genomic DNA was digested overnight at 37°C using *Spe*I and *Bam*HI. Hybridisation and detection of probe was carried out using a non-radioactive, DIG Luminescent Detection Kit for nucleic acids (see section 3.3.5). The full length *luc* gene (1656 bp) was used to synthesise the probe using primer pair Luc-BamHI F and Luc-SpeI R2 (Appendix B.5). Any *A. tumefaciens* contamination in plant tissues was detected using primer pair VCF and VCR (see section 4.3.7).

5.3.9 Dehydration treatment

Dehydration stress was induced by withholding water for eight days. Stressed maize leaves were harvested at 0, 3, 6 and 8 days. Leaf samples were immediately frozen in liquid nitrogen upon collection and thereafter stored at −70°C. Dehydration treatment was done on independent T_1 maize leaf samples.

5.3.10 Analysis of luciferase activity and determination of RWC

The relative water content of the maize plants was calculated (see section 4.3.9). Protein was extracted from dehydrated independent T_1 maize leaf samples and luciferase activity measured (see section 3.3.7).

5.3.11 Total RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA for each treatment was extracted from T_1 maize leaf samples using the TRIzol method (see section 3.3.8). A one step cDNA synthesis using random hexamers was conducted (see section 3.3.9). The qRTPCR was performed using the SYBR green method. Gene specific primers qRTLuc F1 and qRTLuc R2 (Appendix B.5) were used. The 18S rRNA was used as the internal control. The data were normalised and analysed (see section 3.3.10).

5.4 Results

5.4.1 Transformation and selection of putative maize transformants

Following infection, the immature embryos were transferred onto co-cultivation media (Fig. 5.1 A). Browning of the infected immature embryos was observed especially when the infection period was elongated. Visible calli appeared within 5-10 days on resting media. However, longer incubation on resting media resulted in elongated radicles (Fig. 5.1 B). Under selection media lacking bialaphos, growth of non-transformed calli was sustained (Fig. 5.1 C). The selection media containing 3 mg/l bialaphos inhibited the growth of non-transformed embryos (Fig. 5.1 D). Transformed embryos formed compact, opaque, white to pale yellow embryogenic calli on selection media containing 3 mg/l bialaphos (Fig. 5.1 E). However, when transferred to regeneration media in light, the colour of embryogenic calli initially turned green followed by root and shoot development within 7-14 days (Fig. 5.1 F).

5.4.2 Acclimatisation, growth and development of the putative maize transformants

Acclimatisation was successful as all the putative transformants survived the process (Fig. 5.2 A). The transformants grew normally without noticeable abnormal traits (Fig. 5.2 B). The putative transformants were self-pollinated (Fig. 5.2 C) and the ears were covered by pollination bags to maturity (Fig. 5.2 D). The seeds were harvested thereafter. However, tassel seeds were observed (Fig. 5.2 E). A total of 13 putative transformants were generated from three transformation events with a transformation efficiency of 2.3% (Table 5.1).

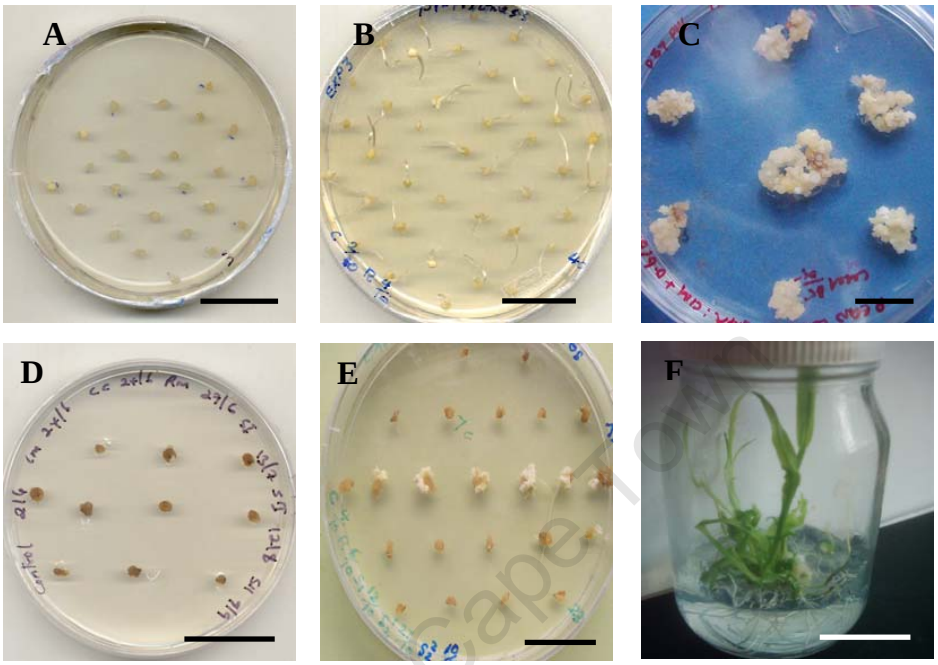

Figure 5.1: Transformation and selection of putative immature zygotic *Z. mays* transformants. **A**: Transformed immature zygotic maize embryos on co-cultivation media. **B**: Transformed immature zygotic maize embryos on resting media lacking bialaphos. **C**: Non-transformed calli 4 weeks after initiation on selection II media lacking bialaphos. **D**: Non-transformed immature zygotic embryos (negative control) 4 weeks after infection showing total necrosis on selection II media containing 3 mg/l bialaphos. **E**: Transformed maize calli 4 weeks after infection showing partial necrosis on selection II media. **F**: Shoots and roots developed on hormone-free regeneration media (scale bars: **A-E** = 2.5 cm; **F** = 3 cm).

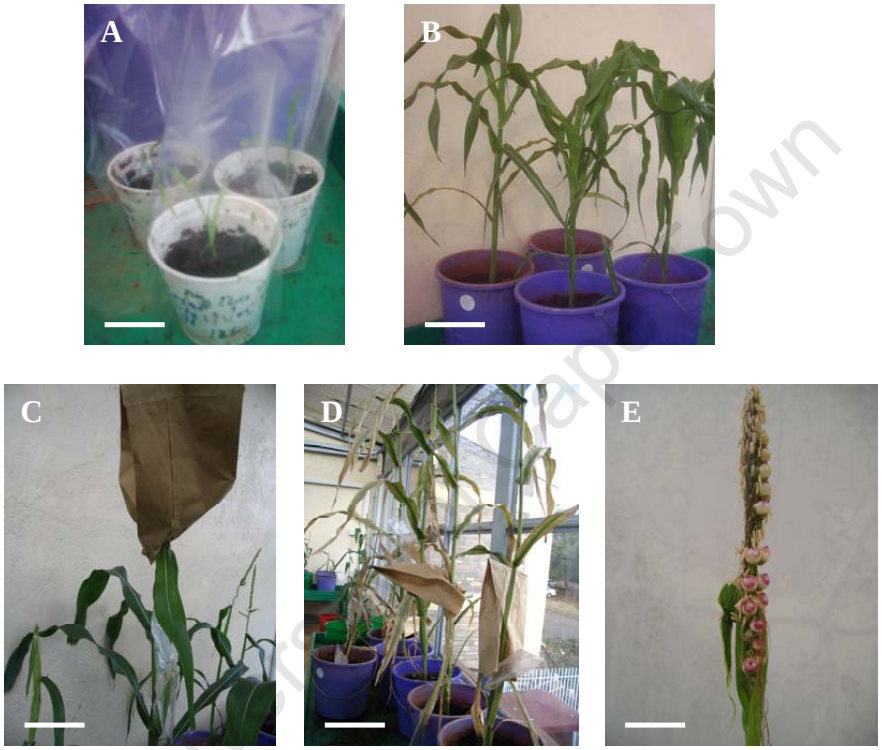

Figure 5.2: Acclimatisation, growth and development of putative *Z. mays* transformants. **A**: Putative maize transformants covered with polythene bags to minimize water loss during hardening. **B**: Putative maize transformants in pots containing loam soil mixed with sand and phytomix growing to flowering. **C**: Putative maize transformants covered with pollination bags to control cross pollination. **D**: Ears of the putative maize transformants covered after pollination to develop seeds. **E**: Putative maize transformants showing tassel seed formation (scale bars: **A** = 5 cm; **B, C, E** = 10 cm; **D** = 2 cm).

Table 5.1: Transformation efficiency of *Z. mays* using XvPsap1::luc construct

No. of transformation events	No. of infected embryos	No. of positive transformants	Transformation efficiency	Average transformation Efficiency ± SE*
I	240	5	2.1	
II	320	2	0.6	
III	140	6	4.3	2.3±1.8 [a]

*SE: Standard deviation of the transformation events

[a]: No significant difference among the transformation events, $P < 0.05$

5.4.3 Basta screening of putative maize transformants and PCR detection

All transformed maize seeds germinated in hydrated vermiculite (Fig. 5.3 A). The seedlings were successfully transferred to pots containing soil prior to basta treatment (Fig. 5.3 B). Non-transformed maize seedlings were observed to be susceptible to basta screening (Fig. 5.3 C) whereas transformed plants displayed tolerance (Fig. 5.3 D). The presence of the *luc* transgene was detected by amplifying the expected 1.2 kb fragment of the gene (Fig. 5.4 A). All transformants that tested positive for the *luc* transgene also showed the presence of the *bar* transgene (Fig. 5.4 B).

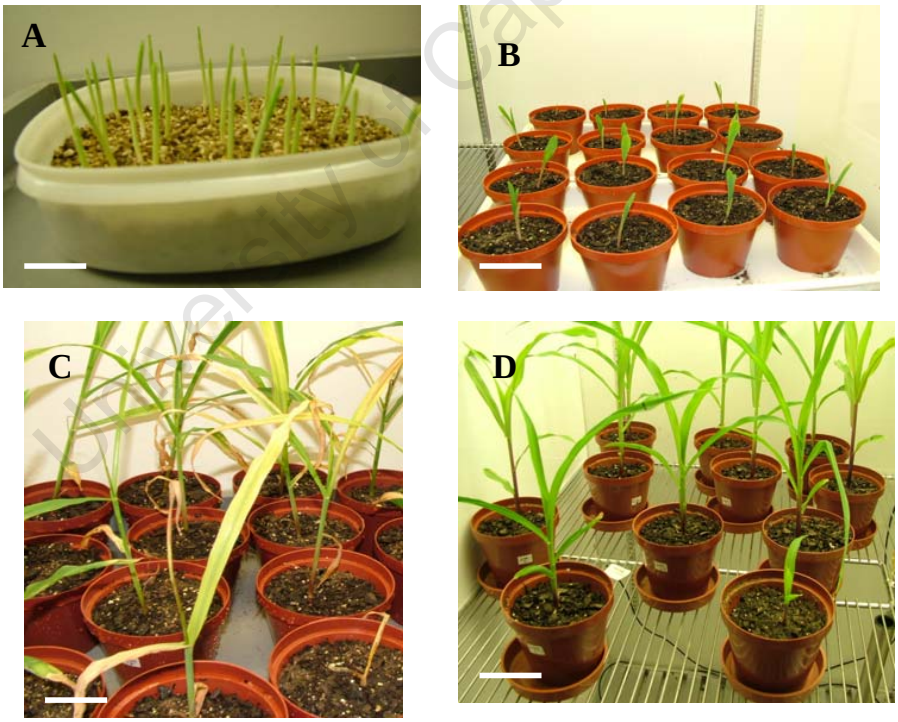

Figure 5.3: Germination and basta screening of putative maize transformants. **A**: Transformed maize seeds germinated on moist vermiculite. **B**: Transformed maize seedlings transferred to pots containing loam soil mixed with sand and phytomix. **C**: Non-transformed maize seedlings susceptible to basta. **D**: Putative maize transformants tolerant to basta (scale bars: **A** = 5 cm; **B-D** = 10 cm).

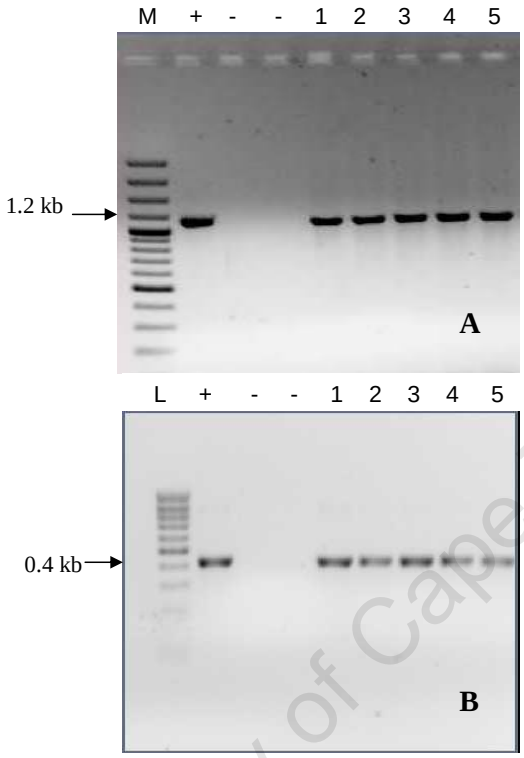

Figure 5.4: Screening of putative transgenic *Z. mays* for the presence of *luc* (**A**) and *bar* transgenes (**B**) by PCR amplification. The DNA used as template was extracted from leaf tissue. **A**: The expected 1.2 kb fragment of the *luc* transgene was amplified. **M**: 100 pb DNA ladder plus (Fermentas, Canada). **+**: 1 ng of pTF101.1 containing the *luc* gene. **-**: non-transformed *Z. mays*. Lanes 1-5, *Z. mays* transformed with XvPsap1 construct. **B**: The expected 421 bp fragment of the *bar* transgene was amplified. **L**: 100 bp DNA ladder (Fermentas, Canada).

5.4.4 Southern blot, luciferase and qRTPCR analyses

The digested genomic DNA was successfully electrophoresed on a 1% agarose gel following digestion with *Spe*I and *Bam*HI (Fig. 5.5 A). Capillary transfer onto the nylon membrane was successful (Fig. 5.5 B). The expected single band (1645 bp) representing the *luc* transgene was observed in all the transformed plants. The non-transformed plants showed no hybridisation product. Transgenics with similar copy numbers were predicted by comparing the band intensities and patterns.

The transgenic maize displayed mild luciferase activity (2.2-fold; P< 0.05; Fig. 5.6). However, qRTPCR demonstrated a significant peak in *luc* expression (4.5-fold; P< 0.05; Fig. 5.7) following three days of dehydration stress.

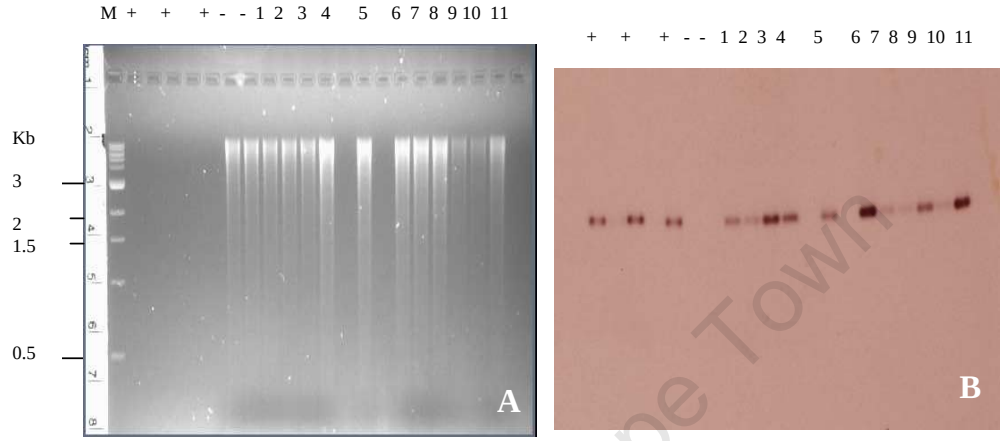

Figure 5.5: Southern blot analysis of transgenic *Z. mays* for the presence of the *luc* transgene. **A**: Digested genomic DNA separated on a 1% agarose gel. **B**: Autoradiograph of membrane probed with a DIG-labeled probe specific to the *luc* gene. **M**: 1 kb DNA ladder (New England Biolabs, USA). Plants transformed with XvPsap1 construct (lanes 1-11). **+**: 200 pg of the full length PCR *luc* gene (1656 bp) used to synthesise the probe. **-**: genomic DNA of non-transformed maize.

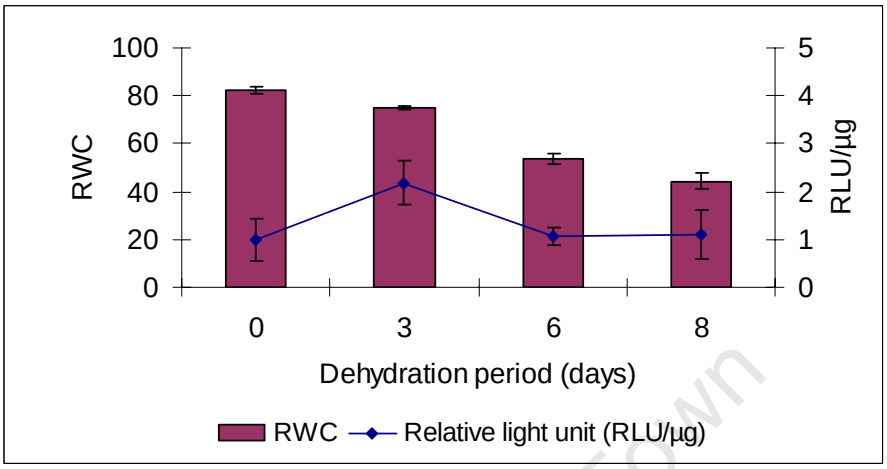

Figure 5.6: Luciferase activity in transgenic maize plants under dehydration stress. The RWC (bars) is plotted on the primary axis and the luciferase activity (line) is plotted on the secondary axis. Luciferase activity was expressed as relative light units (RLU)/μg of protein. The data are presented as means ± SEM from three independent maize transformants.

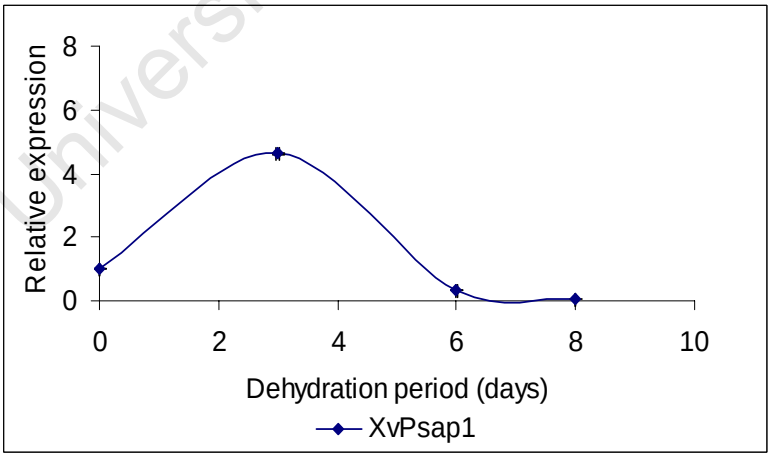

Figure 5.7: Expression profile of *luc* transcripts in transgenic maize following dehydration treatment. The data are presented as means ± SEM from three independent maize transformants

5.5 Discussion

In the present study a transformation efficiency of 2.3% was achieved. These results are consistent with the findings of Frame et al. (2002) who reported a transformation efficiency that ranged from 1.1% to 22.2% in transgenic maize generated by *Agrobacterium*-mediated transformation. However, these values are still low given that higher transformation efficiencies greater than 50% have been reported for other monocots such as rice (Ali et al. 2007) and wheat (Wu et al. 2008). This observation is attributable to the fact that the transfer of T-DNA from bacterium to plant cell is a tightly regulated process and multiple factors from both plant and bacterial cells are simultaneously required for the transformation process (Tzfira & Citovsky 2002). Parameters for the optimal activity of *Agrobacterium* such as acidic pH (Turk et al. 1991), the presence of phenolic inducers such as acetosyringone (Hei et al. 1999) and sugar sources (Cangelosi et al. 1990) were used in this study. However, variations in *Agrobacterium*-mediated transformation efficiency has been reported to be genotype- as well as strain-dependent (Ali et al. 2007). For instance, *Agrobacterium* strain EHA105 is reported to exhibit maximal activity at pH 6.0 irrespective of explant genotype (Ali et al. 2007).

The low transformation efficiency reported in this study can additionally be attributed to the fact that no multiple *vir* genes were used in the helper plasmid. Recently, Wu et al. (2008) reported that the presence of an additional *vir*G gene in pAL155 and *vir*G, *vir*B, *vir*C in pAL154 had beneficial effects, both on T-DNA delivery or transient expression and on the numbers of stable transgenic wheat plants obtained.

Shortly after transfer onto regeneration media, somatic embryos initially turned green prior to the development of shoots and roots. Germination of somatic embryos has been reported to be accompanied by the up-regulation of a number of stress response and membrane transporter genes, and that greening is associated with the up-regulation of many genes encoding photosynthetic and chloroplast

components (Che et al. 2006).

The presence of bialaphos in the regeneration media appeared to slow the regeneration of the transformed maize calli. Consequently, regeneration media lacked bialaphos in this study. This observation emphasises the fact that phosphinothricin inhibits photosynthesis in plants (White et al. 1990). Although transformed maize calli possessed phosphinothricin acetyl transferase enzyme and are therefore expected to detoxify bialaphos, the stress imposed by bialaphos may have contributed to the slow regeneration process observed.

The activity of the XvPsap1 promoter in whole maize was assessed in the present study. The fact that the activity peaked shortly after initiating dehydration stress strongly suggests the involvement of the promoter in early responses to drought stress. Furthermore, these results are consistent with the XvPsap1 activity observed in transgenic BMS cells (chapter 3) and tobacco (chapter 4).

Whereas growth and development of maize transformants did not display abnormal traits, tassel seeds were occasionally observed. This phenomenon could be associated with the fact that tissue culture could result in both epigenetic and heritable variation (Larkin & Scowcroft 1981, Jain 2001). Moreover, activation of transposons (Hirochika et al. 1996), DNA amplification, point mutations, altered methylation patterns (Pereza-Echeverria et al. 2001) have been shown to contribute to variation among tissue culture generated plants (Phillips et al. 1994). Furthermore, in spite of rigorous selection for transgenic cells during transformation and regeneration, chimeric plants carrying transgenic and non-transgenic tissues have been reported to persist in T_0 generation (Berthomieu et al. 1994) especially in transgenics generated by shoot tip transformation procedures. The magnitude and type of variation that arise due to somaclonal variation are usually unpredictable and not strictly controllable (Karp 1995).

Most variations observed among transgenics relate to the traits that are directly expected to be influenced by the transgene (Bhat & Srinivasan 2002). However,

in the present study the transgenic plants that formed tassel seeds did not display other unwanted phenotypes such as dwarfism commonly associated with transgenic plants containing constitutive promoters. This observation strongly points to the fact that the XvPsap1 is indeed stress-inducible.

It is important to note that gene expression in an organism is influenced by a complex interaction of external factors and internal products. Therefore, transgene expression reported in this study cannot be viewed in isolation. However, in light of these results, the XvPsap1 promoter could therefore find application in the generation of transgenic drought tolerant monocot plants.

Chapter 6

General Discussion

Yield losses of major cereals such as maize, rice and wheat caused by abiotic stresses represent a significant economic and political factor and contribute to food shortages in many under-developed countries. In sub-Saharan Africa, it is estimated that one third of all children go to bed hungry and have their mental and physical development compromised by poor nutrition (Rosegrant et al. 2001, Toenniessen et al. 2003).

The application of biotechnology within the agricultural sector can potentially improve food security by making crops more tolerant to adverse weather and soil conditions thus improving yields. According to Abdalla et al. (2003), the potential gains from the uptake of biotechnology are highest for developing countries, particularly those in the low income regions.

With the yield losses attributed to drought stress increasing, so is the need to develop transgenic drought tolerant crops. In order to avoid the unwanted phenotypes commonly observed when transgenes are constitutively expressed, use of stress-inducible promoters is therefore desirable. Whereas the application of stress-inducible promoters such as *rd29A* from *Arabidosis* (Kasuga et al. 2004, Shiqing et al. 2005, Pino et al. 2007), ABRC from barley (Lee et al. 2003) and SWPA2 from sweetpotato (Kim et al. 2003) have been reported, the current knowledge and availability of stress-inducible promoters is still insufficient.

The results reported here not only strongly advance the search for a novel stress-inducible promoter but also meet the primary objective of this study, of assessing the XvPsap promoter activity in different plant systems. The novelty of the promoter was confirmed as no significant similarity with any known plant promoter was observed. The presence of various *cis*-acting elements usually associated with abiotic stress responsiveness was predicted and various constructs were generated.

To augment such computational predictions, the activity of the XvPsap promoters in BMS suspension cells was assessed. The XvPsap1 fragment was revealed to be the most active. It was postulated that the promoter activity observed in BMS cells would not apply for whole plants. Importantly, the activity of the XvPsap promoter in a dicot system would extend its application regarding crop improvement. As a result, the activity of the XvPsap promoters was assessed in tobacco. The XvPsap1 fragment was observed to be the most active. Given that this study is part of a larger project that seeks to genetically engineer maize for drought tolerance, analysis of the XvPsap promoters in maize is imperative. Since, analysis of the promoter fragments in transgenic BMS cells and *N. tabacum* revealed XvPsap1 as the most active of the three promoter fragments, the XvPsap1 fragment was selected and used to transform maize. The XvPsap1 promoter was observed to be both active and stress-inducible. The results reported in this study emphasise the potential utility of the XvPsap1 promoter with respect to improving crops such as maize for drought tolerance.

In conclusion, genetic transformation has the potential to play a significant role in more rapidly advancing agricultural productivity in developing countries while protecting the environment for future generations. Accordingly, and in a separate study, generation of drought tolerant transgenic maize has been initiated, using constructs containing genes postulated to confer drought tolerance regulated by the XvPsap1 promoter.

6.1 Recommendations for future work

The presence of a *cis*-acting element involved in salicylic acid responsiveness suggests that the XvPsap1 promoter may be involved in biotic stress response. Salicylic acid is recognised as an endogenous signal, mediating in plant defense against pathogens by inducing the production of pathogenesis-related proteins (Galis & Matsuoka 2007). To extend the application of the XvPsap1 promoter to include generation of transgenic crops tolerant to biotic stress, analysis of the XvPsap1 promoter activity following biotic stress needs to be assessed.

Stress-inducible promoters usually present the limitation of low activity. Therefore specific nucleotide deletions or insertions aimed at creating additional known *cis*-acting regulatory sites needs to be performed in order to enhance the activity of the XvPsap1. Such manipulations would create new binding sites for a variety of transcription factors thus widening the application of the XvPsap1 promoter. Furthermore, the activity of XvPsap1 promoter compared to other existing inducible promoters such as Rd29A needs to studied. Inducibility of XvPsap1 promoter under light and MeJA also calls for further studies.

One of the criticism of the first-generation transgenic plants is the application of antibiotic resistant genes for selection thus presenting the potential risk of gene flow. The new transplastomic crops (Ahmadabadi et al. 2007, De Marchis et al. 2009) are likely to be free of this undesirable trait, as there already exit various protocols for the production of marker-free transplastomic plants (Maliga & Small 2007). Since consumer acceptance in the market place is the bottom line, future work involving the XvPsap1 promoter should employ such new technologies in order to address sensitive issues related to the risks perceived by the consumers, which slows the process of commercialisation of genetically modified organisms. Moreover, generation of cisgenics involving the transfer of genes from the same species should also be explored in order to minimise resistance to genetically modified organisms.

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Appendices

Appendix A

General protocols and media

A.1 PCR product purification

Amplified DNA was purified using the EZ-10 Spin Column PCR product purification Kit (Bio Basic, Canada). The PCR product was transferred to a 1.5 ml microfuge tube and 3 volumes of Binding Buffer I added. After pulsing, the mixture was transferred to the column and let to stand for 2 min at room temperature before spinning for 1 min at 5,000g. The flow-through was discarded and the column placed back on the same collection tube. Five hundred microlitres Wash Buffer was added to the column and centrifuged for 1 min at 8,000g. The flow-through was discarded and the column again combined with the collection tube. The washing step was repeated with centrifugation for 60 s of 10,000g. To remove the residual Wash Solution, the above step was repeated. The collection tube was discarded and the column inserted into a sterile 1.5 ml microfuge tube. To the center part of the column, 40 μl Elution Buffer was added and incubated at room temperature for 2 minutes before centrifuging at 10,000g for 1 min. For DNA intended for cloning, water was used for elution. The purified DNA was stored at 4°C for regular use or at -20°C for long term storage.

A.2 DNA puification from agarose gels

Excised DNA fragments from agarose gels were purified using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada). Upon excision of the band of interest from the agarose gel, the gel slice was placed in a sterile previously weighed 1.5 ml Eppendorf tube and the mass estimated. For every 100 mg of excised agarose, 300 μl of Binding Buffer II was added to the Eppendorf tube. The tube was vortexed for 45 s to resuspend the gel slice in Binding Buffer II. The suspension was incubated for 10 min at 55°C and vortexed briefly every 2-3 min during this period.

The mixture solution was added to the spin column and let to stand for 2 min at room temperature before centrifuging for 1 min at 5,000g. The flow-through was discarded and the column placed back on the same collection tube. Five hundred microlitres of Wash Buffer was added to the column and centrifuged for 1 min at 8,000g. The flow-through was discarded and the column again combined with the collection tube. Five hundred microlitres of Wash Buffer was added and the sample centrifuged for 1 min at 10,000g. The above step was repeated. The collection tube was discarded and the column inserted into a sterile 1.5 ml microfuge tube. To the center part of the column, 40 μl Elution Buffer was added and incubated for 2 min at room temperature before centrifuging for 1 min at 10,000g. The purified DNA was stored at 4°C for regular use or at -20°C for long term storage.

A.3 Plasmid DNA isolation

Plasmid DNA was isolated using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic, Canada). To the 1.5 ml microfuge, 1.5 ml of overnight culture was added and centrifuged for 15 s at 10,000g. The supernatant was completely drained, 100 μl Solution I added to the pellet and gently mixed at room temperature.

Two hundred microlitres of Solution II was added to the mixture, gently mixed

by inverting 6 times then allowed to stand for 5 min on ice. Three hundred and fifty microlitres of Solution III was added to the mixture and gently mixed before incubating for 5 min on ice. The mixture was centrifuged for 15 min at 12,000g. Thereafter the supernatant was transferred to the column, allowed to stand for 2 min then centrifuged for 1 minute at 5,000g.

The flow-through was discarded and the column again combined with the collection tube. Five hundred microlitres of Wash Buffer was added and the sample centrifuged for 1 min at 8,000g. The above step was repeated. Further centrifugation for 30 s at 10,000g was performed to remove the residual wash buffer. The collection tube was discarded and the column inserted into a sterile 1.5 ml microfuge tube. To the center part of the column, 40 μl double distilled water was added and incubated for 2 min at 37°C before centrifuging for 1 min at 10,000g. The purified plasmid DNA was stored at 4°C for regular use or at −20°C for long term storage.

A.4 Preparation of glycerol stocks

Following overnight culture, cells were pelleted down for 1 min at 3,000g. Two hundred microlitres of autoclaved 50% glycerol was added and gently mixed. The glycerol stocks were then stored at −80°C.

A.5 Controlled pollination of maize

Growth and development of maize plants was closely monitored to mark the sprouting ears. The shootbags were tightly secured on the emerging top ear of the plant to cover the shoot before the silk emerged to prevent pollen contamination. A day before pollination, ears were cut to ensure growth of fresh silk for pollination. The shootbags were promptly placed over the freshly cut ears to protect them. To indicate which ears were to be pollinated the following day, the top of the shootbags

were folded away from the ears. The plant's tassels were routinely checked to ensure that the plants would shed enough pollen for pollination. After identifying the mature tassels, pollination bags were carefully placed over the plant's tassels and the tassels grasped by holding the bag and making a central ridge for better support. The bag's flaps were then secured with non-skid paper clip. On the day of pollination, the bags were tapped five to seven times to release pollen from the tassels. The bags were carefully taken down to avoid contamination from other pollen sources by keeping shut the opening of the bag. The shootbags were pulled out while covering the shoot with the pollination bag to prevent the silk from getting exposed. The bags were straightened and tapped slightly to allow the pollen to be introduced to the fresh silk. All the four flaps of the bags were finally tightly stapled against the stalk. This protocol was adopted from Schnable Laboratory of Iowa state University http://schnablelab.plantgenomics.iastate.edu/resources/pollination/

A.6 Embryo dissection

Following sterilization, a long forceps was inserted into the top of the ears to act as a handle. The top half of kernels were cut off while still attached to the sterilized ears using a scalpel. With a spatula, the endosperm was scooped meticulously and discarded leaving the embryo, which could be gently removed with the spatula under aseptic conditions. These manipulations were carried out in a sterile larmina flow chamber using sterile forceps and scalpels sterilized with 70% alcohol or methylated spirit followed by flaming.

A.7 LB Media

All components were mixed and the pH adjusted to 7.0 and autoclaved for 20 min at 121°C and 15 psi. The appropriate antibiotic was filter sterilised and added prior to use. For blue-white screening for positive clones, X-gal and IPTG were

added to the LB Agar.

Table A.1: LB Media

Components	LB Agar (1l)	LB Broth (1l)
Tryptone	10 g/l	10 g/l
Yeast extract	5 g/l	5 g/l
NaCl	5 g/l	5 g/l
Agar	15 g	-
dH_2O	Add upto 1l	Add upto 1l

Appendix B

Standard protocols, vector maps, primers and PCR cycling conditions, promoter and gene sequences

B.1 Standard protocols

Table B.1: PCR reagents and final volumes used in a standard PCR protocol

Components	Final volume
Forward primer (10 μM)	1 μl
Reverse primer (10 μM)	1 μl
dNTPs (2.5 mM)	2.5 μl
Buffer (10X)	2.5 μl
$MgCl_2$ (25 mM)	1.5 μl
dH_2O	15.3 μl
Supertherm *Taq* (5U/μl)	0.5 μl
Template (10 ng/μl)	1 μl

Table B.2: Reagents and final volumes used in a standard double digestion reaction

Components	Final volume
Plasmid DNA (400 ng)	x μl
Enzyme I (2U/μl)	1 μl
Enzyme II (2U/μl)	1 μl
Appropriate Buffer (10X)	2 μl
dH_2O	(16 - x) μl

Table B.3: Reagents and final volumes used in a standard ligation protocol

Components	Final volume
Vector DNA (400 ng)	x μl
Insert DNA (2 μg)	y μl
T4 DNA ligase (2U/μl)	1 μl
Ligation Buffer (10X)	1 μl
dH_2O	(8 - x - y) μl

Table B.4: Standard ligation-reaction mixture using pDrive cloning vector

Component	Volume/reaction
pDrive Cloning Vector (50ng/μl)	1μl
PCR product	1-4μl
Distilled water	variable
Ligation Master mix (2X)	5μl
Total	10μl

B.2 Vector maps

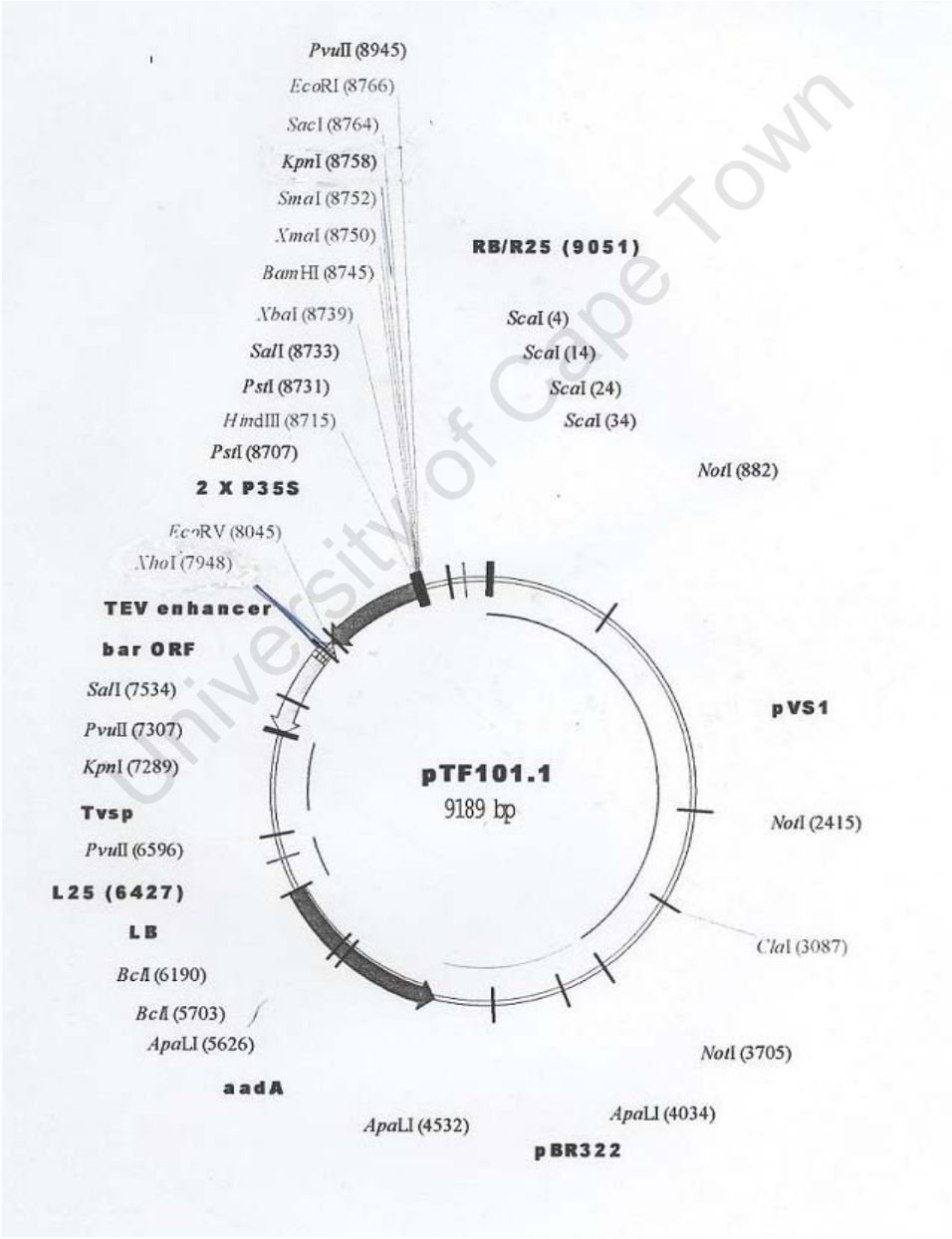

Figure B.1: pTF101.1 vector

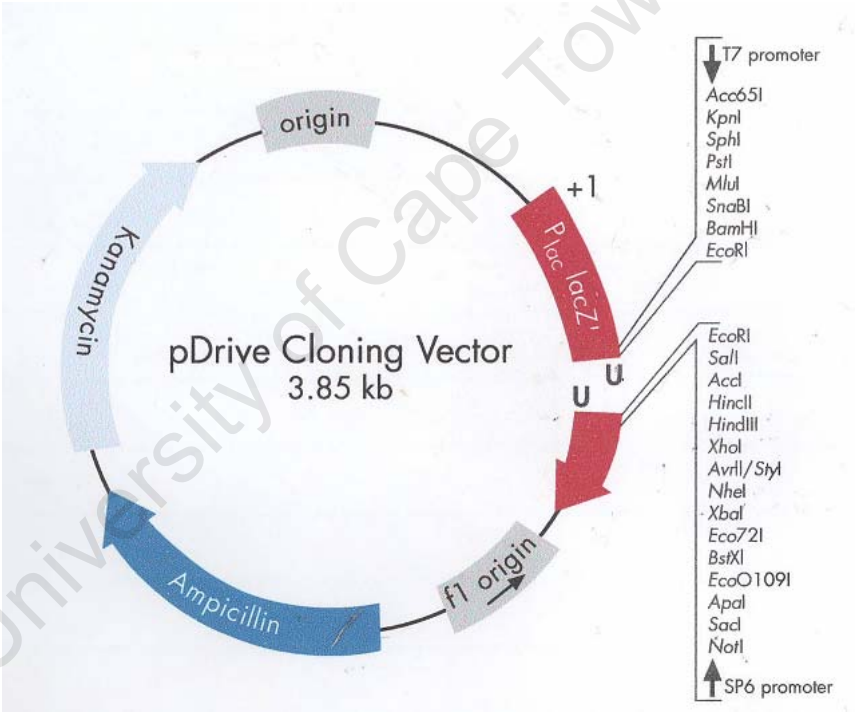

Figure B.2: pDrive Cloning Vector

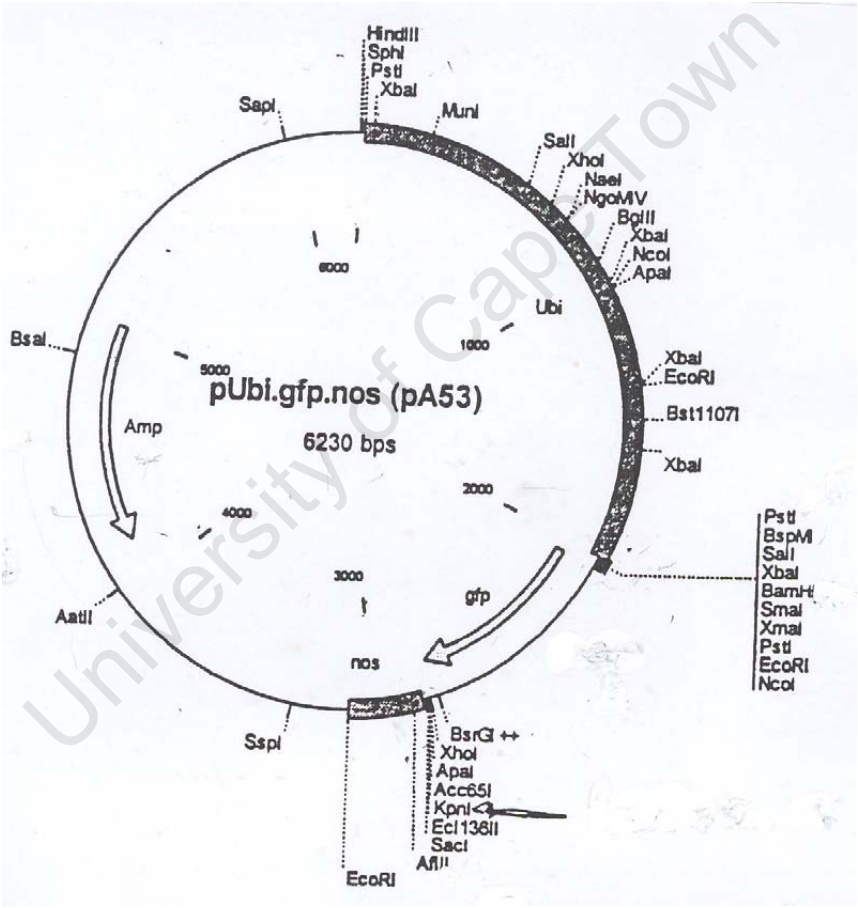

Figure B.3: pA53 plasmid used for biolistics transformation

B.3 Primer combinations and cycling conditions

Table B.5: Primer sequences used for PCR and qRTPCR amplification including the cycling conditions

Primer name	Target sequence	Primer sequence* $5' \rightarrow 3'$	Product size (bp)	Cycle conditions
SAP-prom-EcoRI-F_1	XvPsap1	GGAATTCACTGTCTGGGTAGCTGG	2083	94^oC, 60s;
SAP-prom-BamHI-R_2		TCCGGATCCTCCCTAATATCTCTCGCTC		58^oC, 30s;
				72^oC, 90s
SAP-prom-EcoRI-F_2	XvPsap2	GGAATTCCGTCACTTTCCATGGG	1577	94^oC, 60s;
SAP-prom-BamHI-R_2		TCCGGATCCTCCCTAATATCTCTCGCTC		58^oC, 30s;
				72^oC, 90s.
SAP-prom-EcoRI-F_3	XvPsap2	GGAATTCGACCCCCAAAACACC	1127	94^oC, 60s;
SAP-prom-BamHI-R_2		TCCGGATCCTCCCTAATATCTCTCGCTC		58^oC, 30s;
				72^oC, 90s
nos-speI XF2	*nos*	ACTAGTGAATTTCCCCGATCGTTC	300	94^oC, 30s;
nos-hindIII XR1		GAAAGCTTAGGGATCTAGTAACATAGATGAC		57^oC, 30s;
				72^oC, 60 s
Luc-BamHI F	*luc*	GGATCCATGGTCACCGACGCCAAAAAC	1656	94^oC, 30s;
Luc-SpeI R2		GAACTAGTTACACGGCGATCTTTCC		58^oC, 30s;
				72^oC, 90 s
GFP-BamHI F	*gfp*	GGATCCCCATGGTGAGCAA	759	94^oC, 60s;
GFP-speI R		CGACTAGTGCCGCTTTACTT		58^oC, 60s;
				72^oC, 60 s
Bar I	*bar*	GGTCTGCACCATCGTCAACC	421	94^oC, 60s;
Bar II		GTCATGCCAGTTCCCGTGCT		57^oC, 30s;
				72^oC, 90s.
Luc F_2	*luc*	CACGTTCGTCACATCTCATC	1200	94^oC, 60s;

Continued...

Table B.5 – Continued

Primer name	Target sequence	Primer sequence* $5' \rightarrow 3'$	Product size (bp)	Cycle conditions
Luc-SpeI $R2_2$		GAACTAGTTACACGGCGATCTTTCC		58^oC, 30s; 72^oC, 90s.
qRTLuc F1	*luc*	GAGGCGAACTGTGTGTGAGA	192	95^oC, 5s;
qRTLuc R1		GAGCCACCTGATAGCCTTTG		60^oC, 8s; 72^oC, 12 s
qRT18S-F2	*18SrRNA*	CGTTGGCCTTCGGGATCGGAG	200-250	95^oC, 5s;
qRT18S-R2		CATAAGGTGCCGGCGGTGTCC		60^oC, 8s; 72^oC, 12 s
VCF	*virC*	ATCATTTGTAGCGACT	730	95^oC, 60s;
VCR		5'- AGCTCAAACCTGCTTC		55^oC, 60s; 72^oC, 120 s

*The underlined nucleotides in the primer sequences represent the restriction sites

B.4 XvPsap promoter and *luc, nos, gfp* and *XvSap1* sequences

```
1       ACTGTCTGGG TAGCTGGCAA TATAGAGACG TAAGAAATTC ATGGATCATC ACCCTAATTC
61      GGTCTTTCAC TCATTTTATC CTAGACCTGA CTAAAAAACT TGGTCAGAGT TTTACTTATT
121     TAAAAAAAAG AGGACTTCAT GGCATCCATG TGCAGGTACA GCTCCCAGAA AAAAAAAGCA
181     TGAAACACGA CAGGATCAAT AGCATTCGAT CTGAAACAAA AGGTTGGAGC TCAAGACTTT
241     CTCCAAAATA TTAAGATGAT CCAAAGAATT ACCCCAAGAT ATCCAACGTA TACCAATGTG
301     TATACCGAAA GTAAGAAAGT TCACGTGCAT TCTTTGATTT TTCTCCCGAG TGTTCTTTTC
361     TGAAATGAGT AAATAAGACT AGAATAAGAG CTAATGTATT TTTTTTCTAA AAAAAAGTTG
421     AATGTGGATA CAATATGATT ATACATTCAT TAGCTATTTT AAGTATATTC TATTTTTTTT
481     CCCCCCAAAA GAACACAAAT GTGTTCCGTC ACTTTCCATG GGGCAAATTA CAACTTAGGC
541     TTTATCTTAG TTGGTATGAT CTTAATTTTA TTATACTTTA AACAACTTAT CGCTAATAAT
601     TTTGTTTTGA TTTATGCGCC AATTGTAAAT ATAAATCGGA TAAATTTGAA CATTAATACT
661     TTTAGTCAGT TTCAAAAAAG AAAAAGATAA CTATGACGTT AGAGTTTGGA ATCCAGTCAA
721     ATGGAACTTA TTTTTTAGTT CATCAGAATC AACTTGATGA GATTTTTTGT ACTAGACAAT
781     CATCTTGAAT GATAGATAGG GGACTTACCA ATCAGCCCCC CATATTTTGA AACTTTCAAC
841     GCGCCCCTCA ACATTTTTCT CTTTCAACGC GCCCTTCCAT CACTTTTCTC TTTTCATCGC
901     CCCTCCCTCA ACTTTTTGGT CGGACGGAAA TACCTCTATA TATATTTCAT CATTTCGACC
961     CCCAAAACAC CTATTTAGAA GTATTTTTTG GAAAAAATTT TAACATGAAA GTTTTAGATC
1021    TTGATGAGAT CTACAATTTT TATGTTGAAA GTTTTTCCAA AAAATACTTT TAGATAGATA
1081    TTTTGGAACT CCGAAGTGTT AATGGGTCGA CCCGCTAACT TGCGGAAATA GAAAAACATC
1141    AAGATCTACA ACTTTTATGT TGAAAGTTTT TTCAAAAAAT ACTTCTAGAT AGATATTTGA
1201    TTTGTAATTT TAATGTTGAA AGTTTTTTCA AAAAATACTT CTAGATAGAT ACTTTGGGGC
1261    TCCGGAGTGT TAACAAGTAT AGGAATATTT TTGTCTGCAA AAAATTAATT TTTCGGACAA
1321    GAGGCCGATC AGTAAGGAAT CTGGTCGGAG GGGCTGTTCG GCAATATAAG TTCAGATAGG
1381    AGAACTGATC GGATATTTTT CCTTAATTTA ATTCCAATTT GATACTATTA AGAATGAAAA
1441    CATCCTAATA ATTGTGACCA CTTTATAGCA CCACATTTAT TTTAATTTAA ATCTTTTAAA
1501    TCTTAGAATT GGACAGGGTG CTTATGATAA CAAACTTGTT CCTATCAACA ACTGCATGTT
1561    AGACAGCGCC GAATTTACAG TCCTACTGGG CGCCACTTTT CAACCCACAT CATCAAGATG
1621    AACACCACGT TATCTTCATC CGCTCCAACC ACATGGTCCA GCGCCACTGG CCAAGACCGC
1681    CAGCCAGCCA GGCCATCCAA CGTGGTGCAT TTTCTAACAC TCCACGTTCG CTGTACGGCA
1741    TTATTTCTCC AGCCAGAAAG ACCGAGACAG CGACGCTGTT GGGCGGGCCC GCGGCCTGCT
1801    CTCTCTGCTT CCCCATGAGA TTCACGGGCA TCGCTCCTCG CTCGTGCCTA CGCCCGCGCC
1861    CGCGCCCGAC CGCGCCGGTC CACGTGACGT GGCGCAGCAA TCGTTCTTAC TAGGCGCTTG
1921    CACGTGTCGT TCGCATGCGA AGCGTCCACA CTGCCAACGA CCTCCTTAAA TATCCTTGTG
1981    ATATTCGCCT TACGATCTCA CACTTCGCAC GCAAAGGCCA GTCGCAGATT TGGGTTGAAT
2041    TTGCTGCGTT TTGGCAGATT TTGAGCGAGA GATATTAGGG AAG
```

Figure B.4: Nucleotide sequence for promoter XvPsap1: 2083 bp (SEQ ID NO: 1); Composition 626 A; 435 C; 367 G; 648 T; 7 other; Percentage: 30.1% A; 20.9% C; 17.6% G; 31.1% T; 0.3% other; Molecular Weight (kDa): ssDNA: 641.55 dsDNA: 1283.94

```
1       CGTCACTTTC CATGGGGCAA ATTACAACTT AGGCTTTATC TTAGTTGGTA TGATCTTAAT
61      TTTATTATAC TTTAAACAAC TTATCGCTAA TAATTTTGTT TTGATTTATG CGCCAATTGT
121     AAATATAAAT CGGATAAATT TGAACATTAA TACTTTTAGT CAGTTTCAAA AAAGAAAAAG
181     ATAACTATGA CGTTAGAGTT TGGAATCCAG TCAAATGGAA CTTATTTTTT AGTTCATCAG
241     AATCAACTTG ATGAGATTTT TTGTACTAGA CAATCATCTT GAATGATAGA TAGGGGACTT
301     ACCAATCAGC CCCCCATATT TTGAAACTTT CAACGCGCCC CTCAACATTT TTCTCTTTCA
361     ACGCGCCCTT CCATCACTTT TCTCTTTTCA TCGCCCCTCC CTCAACTTTT TGGTCGGACG
421     GAAATACCTC TATATATATT TCATCATTTC GACCCCCAAA ACACCTATTT AGAAGTATTT
481     TTTGGAAAAA ATTTTAACAT GAAAGTTTTA GATCTTGATG AGATCTACAA TTTTTATGTT
541     GAAAGTTTTT CCAAAAAATA CTTTTAGATA GATATTTTGG AACTCCGAAG TGTTAATGGG
601     TCGACCCGCT AACTTGCGGA AATAGAAAAA CATCAAGATC TACAACTTTT ATGTTGAAAG
661     TTTTTTCAAA AAATACTTCT AGATAGATAT TTGATTTGTA ATTTTAATGT TGAAAGTTTT
721     TTCAAAAAAT ACTTCTAGAT AGATACTTTG GGGCTCCGGA GTGTTAACAA GTATAGGAAT
781     ATTTTTGTCT GCAAAAAATT AATTTTTCGG ACAAGAGGCC GATCAGTAAG GAATCTGGTC
841     GGAGGGGCTG TTCGGCAATA TAAGTTCAGA TAGGAGAACT GATCGGATAT TTTTCCTTAA
901     TTTAATTCCA ATTTGATACT ATTAAGAATG AAAACATCCT AATAATTGTG ACCACTTTAT
961     AGCACCACAT TTATTTTAAT TTAAATCTTT TAAATCTTAG AATTGGACAG GGTGCTTATG
1021    ATAACAAACT TGTTCCTATC AACAACTGCA TGTTAGACAG CGCCGAATTT ACAGTCCTAC
1081    TGGGCGCCAC TTTTCAACCC ACATCATCAA GATGAACACC ACGTTATCTT CATCCGCTCC
1141    AACCACATGG TCCAGCGCCA CTGGCCAAGA CCGCCAGCCA GCCAGGCCAT CCAACGTGGT
1201    GCATTTTCTA ACACTCCACG TTCGCTGTAC GGCATTATTT CTCCAGCCAG AAAGACCGAG
1261    ACAGCGACGC TGTTGGGCGG GCCCGCGGCC TGCTCTCTCT GCTTCCCCAT GAGATTCACG
1321    GGCATCGCTC CTCGCTCGTG CCTACGCCCG CGCCCGCGCC CGACCGCGCC GGTCCACGTG
1381    ACGTGGCGCA GCAATCGTTC TTACTAGGCG CTTGCACGTG TCGTTCGCAT GCGAAGCGTC
1441    CACACTGCCA ACGACCTCCT TAAATATCCT TGTGATATTC GCCTTACGAT CTCACACTTC
1501    GCACGCAAAG GCCAGTCGCA GATTTGGGTT GAATTTGCTG CGTTTTGGCA GATTTTGAGC
1561    GAGAGATATT AGGGAAG
```

Figure B.5: Nucleotide sequence for promoter XvPsap2: 1577 bp (SEQ ID NO: 2); Composition 446 A; 347 C; 285 G; 494 T; 5 other; Percentage: 28.3% A; 22.0% C; 18.1% G; 31.3% T; 0.3% other; Molecular Weight (kDa): ssDNA: 485.37 dsDNA: 972.07

```
1       GACCCCCAAA ACACCTATTT AGAAGTATTT TTTGGAAAAA ATTTTAACAT GAAAGTTTTA
61      GATCTTGATG AGATCTACAA TTTTTATGTT GAAAGTTTTT CCAAAAAATA CTTTTAGATA
121     GATATTTTGG AACTCCGAAG TGTTAATGGG TCGACCCGCT AACTTGCGGA AATAGAAAAA
181     CATCAAGATC TACAACTTTT ATGTTGAAAG TTTTTTCAAA AAATACTTCT AGATAGATAT
241     TTGATTTGTA ATTTTAATGT TGAAAGTTTT TTCAAAAAAT ACTTCTAGAT AGATACTTTG
301     GGGCTCCGGA GTGTTAACAA GTATAGGAAT ATTTTTGTCT GCAAAAAATT AATTTTTCGG
361     ACAAGAGGCC GATCAGTAAG GAATCTGGTC GGAGGGGCTG TTCGGCAATA TAAGTTCAGA
421     TAGGAGAACT GATCGGATAT TTTTCCTTAA TTTAATTCCA ATTTGATACT ATTAAGAATG
481     AAAACATCCT AATAATTGTG ACCACTTTAT AGCACCACAT TTATTTTAAT TTAAATCTTT
541     TAAATCTTAG AATTGGACAG GGTGCTTATG ATAACAAACT TGTTCCTATC AACAACTGCA
601     TGTTAGACAG CGCCGAATTT ACAGTCCTAC TGGGCGCCAC TTTTCAACCC ACATCATCAA
661     GATGAACACC ACGTTATCTT CATCCGCTCC AACCACATGG TCCAGCGCCA CTGGCCAAGA
721     CCGCCAGCCA GCCAGGCCAT CCAACGTGGT GCATTTTCTA ACACTCCACG TTCGCTGTAC
781     GGCATTATTT CTCCAGCCAG AAAGACCGAG ACAGCGACGC TGTTGGGCGG GCCCGCGGCC
841     TGCTCTCTCT GCTTCCCCAT GAGATTCACG GGCATCGCTC CTCGCTCGTG CCTACGCCCG
901     CGCCCGCGCC CGACCGCGCC GGTCCACGTG ACGTGGCGCA GCAATCGTTC TTACTAGGCG
961     CTTGCACGTG TCGTTCGCAT GCGAAGCGTC CACACTGCCA ACGACCTCCT TAAATATCCT
1021    TGTGATATTC GCCTTACGAT CTCACACTTC GCACGCAAAG GCCAGTCGCA GATTTGGGTT
1081    GAATTTGCTG CGTTTTGGCA GATTTTGAGC GAGAGATATT AGGGAAG
```

Figure B.6: Nucleotide sequence for promoter XvPsap3: 1127 bp (SEQ ID NO: 3); Composition 315 A; 256 C; 226 G; 330 T; 0 other; Percentage: 28.0% A; 22.7% C; 20.1% G; 29.3% T; 0.0% other; Molecular Weight (kDa): ssDNA: 347.25 dsDNA: 694.71

```
1       ATGGTCACCG ACGCCAAAAA CATAAAGAAA GGCCCGGCGC CATTCTATCC GCTGGAAGAT
61      GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA
121     ATTGCTTTTA CAGATGCACA TATCGAGGTG GACATCACTT ACGCTGAGTA CTTCGAAATG
181     TCCGTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC
241     GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA
301     GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGGGC
361     ATTTCGCAGC CTACCGTGGT GTTCGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG
421     CAAAAAAAGC TCCCAATCAT CCAAAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG
481     GGATTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC
541     GATTTTGTGC CAGAGTCCTT CGATAGGGAC AAGACAATTG CACTGATCAT GAACTCCTCT
601     GGATCTACTG GTCTGCCTAA AGGTGTCGCT CTGCCTCATA GAACTGCCTG CGTGAGATTC
661     TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT
721     GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA
781     TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTCTGAGGAG CCTTCAGGAT
841     TACAAGATTC AAAGTGCGCT GCTGGTGCCA ACCCTATTCT CCTTCTTCGC CAAAAGCACT
901     CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGTGG CGCTCCCCTC
961     TCTAAGGAAG TCGGGGAAGC GGTTGCCAAG AGGTTCCATC TGCCAGGTAT CAGGCAAGGA
1021    TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG
1081    GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG
1141    AAAACGCTGG GCGTTAATCA AAGAGGCGAA CTGTGTGTGA GAGGTCCTAT GATTATGTCC
1201    GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT
1261    TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATCGTTGA CCGCCTGAAG
1321    TCTCTGATTA AGTACAAAGG CTATCAGGTG GCTCCCGCTG AATTGGAATC CATCTTGCTC
1381    CAACACCCCA ACATCTTCGA CGCAGGTGTC GCAGGTCTTC CCGACGATGA CGCCGGTGAA
1441    CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG
1501    GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG
1561    GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC
1621    ATAAAGGCCA AGAAGGGCGG AAAGATCGCC GTGTAA
```

Figure B.7: Nucleotide sequence for *luc* gene: 1656 bp. Composition 453 A; 366 C; 410 G; 427 T; 0 OTHER Percentage: 27.4% A; 22.1% C; 24.8% G; 25.8% T; 0.0% OTHER Molecular Weight (kDa): ssDNA: 512.26 dsDNA: 1020.87

```
1       GAGTACGGTG GGTAGCCCGA TCGTTCAAAC ATTTGGCAAT AAAGTTTCTT AAGATTGAAT
61      CCTGTTGCCG GTCTTGCGAT GATTATCATA TAATTTCTGT TGAATTACGT TAAGCATGTA
121     ATAATTAACA TGTAATGCAT GACGTTATTT ATGAGATGGG TTTTTATGAT TAGAGTCCCG
181     CAATTATACA TTTAATACGC GATAGAAAAC AAAATATAGC GCGCAAACTA GGATAAATTA
241     TCGCGCGCGG TGTCATCTAT GTTACTAGAT CCCTAGGCTA TCTGTCACTT CATCAAAAGG
```

Figure B.8: Nucleotide sequence for *nos* terminator: 300 bp. Composition 92 A; 50 C; 61 G; 97 T; 0 OTHER Percentage: 30.7% A; 16.7% C; 20.3% G; 32.3% T; 0.0% OTHER Molecular Weight (kDa): ssDNA: 92.80 dsDNA: 184.91

```
1       GAGGATCCCC GGGTACCGGT CGCCACCATG GTGAGCAAGG GCGAGGAGCT GTTCACCGGG
61      GTGGTGCCCA TCCTGGTCGA GCTGGACGGC GACGTAAACG GCCACAAGTT CAGCGTGTCC
121     GGCGAGGGCG AGGGCGATGC CACCTACGGC AAGCTGACCC TGAAGTTCAT CTGCACCACC
181     GGCAAGCTGC CCGTGCCCTG GCCCACCCTC GTGACCACCC TGACCTACGG CGTGCAGTGC
241     TTCAGCCGCT ACCCCGACCA CATGAAGCAG CACGACTTCT TCAAGTCCGC CATGCCCGAA
301     GGCTACGTCC AGGAGCGCAC CATCTTCTTC AAGGACGACG GCAACTACAA GACCCGCGCC
361     GAGGTGAAGT TCGAGGGCGA CACCCTGGTG AACCGCATCG AGCTGAAGGG CATCGACTTC
421     AAGGAGGACG GCAACATCCT GGGGCACAAG CTGGAGTACA ACTACAACAG CCACAACGTC
481     TATATCATGG CCGACAAGCA GAAGAACGGC ATCAAGGTGA ACTTCAAGAT CCGCCACAAC
541     ATCGAGGACG GCAGCGTGCA GCTCGCCGAC CACTACCAGC AGAACACCCC CATCGGCGAC
601     GGCCCCGTGC TGCTGCCCGA CAACCACTAC CTGAGCACCC AGTCCGCCCT GAGCAAAGAC
661     CCCAACGAGA AGCGCGATCA CATGGTCCTG CTGGAGTTCG TGACCGCCGC CGGGATCACT
721     CTCGGCATGG ACGAGCTGTA CAAGTAAAGC GGCCGCGAC
```

Figure B.9: Nucleotide sequence for *gfp* gene : 759 bp. Composition 180A; 256C; 217 G; 106 T; 0 other. Percentage: 23.7% A; 33.7% C; 28.6% G; 14.0% T; 0.0% other. Molecular Weight (kDa): ssDNA: 133.94 dsDNA: 468.02


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1       ATGAGGAACG AGGGTTTTCT GAAAATGAAG ACCGACGTTG GAGTCGCCGA CGAGGTGATC
61      TCCGGAGATC TCAAGCAGCT TGGTGACGCT GCAAAGCGGC TAGCTAAACA TGCGATCAAG
121     CTCGGCGCCA GCTTCGGGGT TGGCTCTACC ATAGTCCAGG CTATTGCTTC GATCGCTGCT
181     ATCTATTTGT TGATATTGGA CCGGACAAAC TGGCGTACAA ATATCTTGAC ATCACTTCTA
241     ATTCCATATG TTTACTTGAG TCTTCCTTCA GTGATATTCA ACCTATTCAG GGGCGACCTG
301     GGCAGATGGC TTTCATTCAT TGGCGTAGTA ATGAAGCTCT TCTTCCACCG ACACTTCCCA
361     GTTACCTTGG AACTGCTTGT GTCTCTCATT CTCCTGATTG TGGTTTCCCC CACTTTCATT
421     GCCCACACAA TCAGAGGCAG TCTCATTGGA GTCTTCATCT TCCTTGTCAT CGCCTGCTAC
481     CTCCTCCAAG AGCACATTAG ATCAGCTGGT GGCTTCAAAA ACGCGTTCAC AAAGAGCAAT
541     GGGATTTCAA ACAGCGTCGG GATCATCATT CTACTGATCC ACCCGATCTG GAGCTTGGTG
601     GTGTATTTCC TCTACACGTC TTTGCTGCAA CTTCTTGCAT ACTCTCCTTC CCCTTGTTGT
661     TGCATATTAT ACAATAAGTG GTTTAATTTC ATGCATGTTT GTAAATGTGT AAGCCTTCAT
721     ATGTATTCTC AGTCAATTGG GTCATGCGTG TCCATATTTT TCGTGCAGTT TGTATTCATC
781     TATGAAGCTG AATTTTAA
```

Figure B.10: Nucleotide sequence for *XvSap1* gene: 798 bp. Composition 180 A; 193 C; 170 G; 255 T; 0 OTHER Percentage: 22.6% A; 24.2% C; 21.3% G; 32.0% T; 0.0%OTHER Molecular Weight (kDa): ssDNA: 245.57 dsDNA: 491.93

Appendix C

XvPsap1 Regulatory elements

C.1 XvPsap1 regulatory motifs

Table C.1: Regulatory elements identified in XvPsap1 promoter using PLANTCARE software (Lescot et al., 2002)

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
ABRE	*A. thaliana*	322	+	6	CACGTG	*cis*-acting element involved in the abscisic acid responsiveness
ABRE	*A. thaliana*	1881	+	6	CACGTG	*cis*-acting element involved in the abscisic acid responsiveness
ABRE	*H. vulgare*	1919	+	9	CGCACGTGTC	*cis*-acting element involved in the abscisic acid responsiveness
ABRE	*A. thaliana*	1887	+	7	ACGTGGC	*cis*-acting element involved in the abscisic acid responsiveness
ABRE	*A. thaliana*	1921	+	6	CACGTG	*cis*-acting element involved in the abscisic acid responsiveness
A-box	*P. crispum*	922	-	6	CCGTCC	*cis*-acting regulatory element
ACE	*P. crispum*	391	+	9	CTAACGTATT	*cis*-acting element involved in light responsiveness
ACE	*P. crispum*	1697	-	9	ACTACGTTGG	*cis*-acting element involved in light responsiveness
ACE	*P. crispum*	1879	-	7	ACGTGGA	*cis*-acting element involved in light responsiveness
ACE	*P. crispum*	1721	-	7	ACGTGGA	*cis*-acting element involved in light responsiveness

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
ATCT-motif	*Z. mays*	1338	+	9	AATCTGATCG	part of a conserved DNA module involved in light responsiveness
Box 4	*P. crispum*	652	+	6	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box 4	*P. crispum*	1304	+	6	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	*P. sativum*	670	+	7	TTTCAAA	light responsive element
Box I	*P. sativum*	826	-	7	TTTCAAA	light responsive element
Box I	*P. sativum*	1170	+	7	TTTCAAA	light responsive element
Box I	*P. sativum*	1226	+	7	TTTCAAA	light responsive element
Box II	*P. hortense*	1879	+	9	TCCACGTGGC	part of a light responsive element
Box III	*P. sativum*	75	-	9	CATTTACACT	protein binding site
CATT-motif	*Z. mays*	202	+	6	GCATTC	part of a light responsive element
CATT-motif	*Z. mays*	327	+	6	GCATTC	part of a light responsive element
CAAT-box	*G. max*	1034	+	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*G. max*	1450	-	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*H. vulgare*	1362	+	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*H. vulgare*	1898	+	4	CAAT	common *cis*-acting element in promoter and enhancer regions

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
CAAT-box	*B. rapa*	1200	-	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*G. max*	1507	-	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*G. max*	1415	+	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*B. rapa*	2028	-	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*B. rapa*	1195	-	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*H. vulgare*	1451	-	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*A. thaliana*	1414	+	5	CCAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*P. hybrida*	1952	+	7	TGCCAAC	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*A. thaliana*	1360	+	6	GGCAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*A. thaliana*	1508	-	5	CCAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*B. rapa*	1417	-	5	CAAAT	common *cis*-acting element in promoter and enhancer regions

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
CAAT-box	*B. rapa*	2039	-	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAT-box	*A. thaliana*	1592	+	6	GCCACT	*cis*-acting regulatory element related to meristem expression
CAT-box	*A. thaliana*	1663	+	6	GCCACT	*cis*-acting regulatory element related to meristem expression
circadian	*L. esculentum*	274	+	9	CAAAGATATC	*cis*-acting regulatory element involved in circadian control
circadian	*L. esculentum*	639	-	6	CAANNNNATC	*cis*-acting regulatory element involved in circadian control
CGTCA-motif	*H. vulgare*	1885	-	5	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness
G-Box	*P. sativum*	1626	+	6	CACGTT	*cis*-acting regulatory element involved in light responsiveness
G-Box	*P. sativum*	1921	+	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*P. sativum*	322	+	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*P. sativum*	1723	+	6	CACGTT	*cis*-acting regulatory element involved in light responsiveness
G-Box	*P. sativum*	1699	-	6	CACGTT	*cis*-acting regulatory element involved in light responsiveness

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
G-Box	*P. sativum*	1881	+	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*S. tuberosum*	146	-	7	CACATGG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*A. thaliana*	322	+	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*Z. mays*	186	+	6	CACGAC	*cis*-acting regulatory element involved in light responsiveness
G-Box	*Z. mays*	1626	+	6	CACGTT	*cis*-acting regulatory element involved in light responsiveness
G-Box	*A. thaliana*	1881	+	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-Box	*A. thaliana*	1879	-	9	GCCACGTGGA	*cis*-acting regulatory element involved in light responsiveness
G-Box	*Z. mays*	1886	-	6	CACGTC	*cis*-acting regulatory element involved in light responsiveness
GATA-motif	*A. thaliana*	1375	+	7	GATAGGA	part of a light responsive element
GATA-motif	*A. thaliana*	1540	-	7	GATAGGA	part of a light responsive element
HSE	*B. oleracea*	759	-	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
HSE	*B. oleracea*	992	+	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
HSE	*B. oleracea*	1047	-	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
HSE	*B. oleracea*	1218	-	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
HSE	*B. oleracea*	1162	-	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
CCGTCC-box	*A. thaliana*	922	-	6	CCGTCC	*cis*-acting regulatory element related to meristem specific activation
CG-motif	*L. gibba*	516	+	8	CCATGGGG	part of a light responsive element
CGTCA-motif	*H. vulgare*	507	+	5	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness
CGTCA-motif	*H. vulgare*	694	-	5	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness
EIRE	*N. tabacum*	654	+	7	TTCGACC	elicitor-responsive element
GATA-motif	*P. sativum*	795	+	7	GATAGGG	part of a light responsive element
I-boxf	*N. plumbaginifolia*	381	-	9	CTCTTATGCT	part of a light responsive element
I-box	*Z. mays*	795	+	7	GATAGGG	part of a light responsive element
LTR	*H. vulgare*	1311	-	6	CCGAAA	*cis*-acting element involved in low-temperature responsiveness

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
MBS	*A. thaliana*	1549	+	6	CAACTG	MYB binding site involved in drought-inducibility
O2-site	*Z. mays*	1605	-	10	GATGATGTGG	*cis*-acting regulatory element involved in zein metabolism regulation
O2-site	*Z. mays*	137	-	9	GATGACATGA	*cis*-acting regulatory element involved in zein metabolism regulation
Sp1	*O. sativa*	1781	+	6	GGGCGG	light responsive element
TGA-element	*B. oleracea*	1926	-	6	AACGAC	auxin-responsive element
TGA-element	*B. oleracea*	1956	+	6	AACGAC	auxin-responsive element
TGACG-motif	*H. vulgare*	1885	+	5	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness
P-box	*O. sativa*	217	-	7	CCTTTTG	gibberellin-responsive element
Skn-1 motif	*O. sativa*	693	-	5	GTCAT	*cis*-acting regulatory element required for endosperm expression
TC-rich repeats	*N. tabacum*	1709	+	9	ATTCTCTAAC	*cis*-acting element involved in defense and stress responsiveness
TCT-motif	*A. thaliana*	1905	+	6	TCTTAC	part of a light responsive element
TGA-box	*G. max*	1885	+	9	TGACGTGGC	part of an auxin-responsive element
TCA-element	*B. oleracea*	353	-	9	CAGAAAAGGA	*cis*-acting element involved in salicylic acid responsiveness
Sp1	*Z. mays*	481	+	5	CC(G/A)CCC	light responsive element
Sp1	*Z. mays*	902	+	5	CC(G/A)CCC	light responsive element

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
Sp1	*Z. mays*	816	+	5	CC(G/A)CCC	light responsive element
Unnamed-1	*G. max*	1403	-	11	GAATTTAATTAA	60K protein binding site
TC-rich repeats	*N. tabacum*	236	+	9	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness
TC-rich repeats	*N. tabacum*	989	-	9	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness
TCT-motif	*A. thaliana*	30	-	6	TCTTAC	part of a light responsive element
TCT-motif	*A. thaliana*	311	-	6	TCTTAC	part of a light responsive element
TGACG-motif	*H. vulgare*	507	-	5	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	*H. vulgare*	694	+	5	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness
TATA-box	*A. thaliana*	19	-	9	TCTATATATT	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	21	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	74	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	92	-	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	110	+	5	TTTTA	core promoter element around -30 of transcription start

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
TATA-box	*A. thaliana*	117	+	8	TATTTAAA	core promoter element around -30 of transcription start
TATA-box	*Z. mays*	119	+	8	TTTAAAAA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	121	-	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*G. max*	249	-	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	289	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*H. annuus*	299	-	6	TATACA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	301	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	408	-	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*B. napus*	438	+	6	ATTATA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	439	-	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	440	+	4	TATA	core promoter element around -30 of transcription start

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
TATA-box	*L. esculentum*	457	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	464	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	566	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*G. max*	569	-	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*B. napus*	570	+	6	ATTATA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	571	-	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	572	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*G. max*	594	+	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	624	+	11	TATAAATATAAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	628	+	9	TATATAAATC	core promoter element around -30 of transcription start
TATA-box	*B. oleracea*	629	+	6	ATATAA	core promoter element around -30 of transcription start

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
TATA-box	*G. max*	654	+	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	660	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	733	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*O. sativa*	765	-	7	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	933	-	9	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	935	+	9	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	937	+	8	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*B. napus*	938	+	6	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	939	+	4	TACAAAA	core promoter element around -30 of transcription start
TATA-box	*B. napus*	940	+	6	ATATAT	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	941	+	4	TATA	core promoter element around -30 of transcription start

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
TATA-box	*L. esculentum*	998	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1012	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1038	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*G. max*	1446	+	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	1278	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1480	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1153	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	1463	-	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	1365	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*G. max*	2073	-	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1068	+	5	TTTTA	core promoter element around -30 of transcription start

Continued...

Table C.1 – Continued

Site name	Organism*	Position	Strand	Matrix score	Sequence	Function
TATA-box	*A. thaliana*	1462	-	6	TATAAA	core promoter element around -30 of transcription start
TATA-box	*B. oleracea*	1364	+	6	ATATAA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1494	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*L. esculentum*	1208	+	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*A. thaliana*	1464	+	4	TATA	core promoter element around -30 of transcription start
TATA-box	*G. max*	1426	-	5	TAATA	core promoter element around -30 of transcription start

Organism*